

How governments should be run

The latest twist in the British government's newest political crisis is less a sign that some people have behaved badly than a proof (if one were needed) that the British constitution needs changing.

WHO would have guessed, two months ago, that the fate of an unsuccessful helicopter manufacturer called Westland (see *Nature* 319, 86; 1986) would have led to the resignation of two senior cabinet ministers and raised serious questions about the survival of the Prime Minister herself? Mr Michael Heseltine, previously Secretary of State for Defence, stormed out of a cabinet meeting on 9 January, complaining that he would be muzzled in his attempts to persuade a European consortium to rescue the nearly bankrupt helicopter company if he were compelled to check his public statements with his colleagues. Now the Secretary of State for Trade and Industry, Mr Leon Brittan, among other things the minister for helicopters, has been compelled to quit the government because of two successive blunders: concealing his knowledge of a letter bearing on the Westland issue from a commercial company (British Aerospace) because it had been marked "confidential" and, then, having taken the initiative, at an earlier stage, in leaking to the press an extract from an indubitably confidential letter, from another of his colleagues to Mr Heseltine, in a way that was bound to seem to damage the Heseltine cause. The Prime Minister, Mrs Margaret Thatcher, has been put in political jeopardy, now or later, because it is not clear whether she conspired in the decision to leak the crucial section of the confidential letter (the rest of which had not been published until Monday).

Few in Britain seem to recognize that this heady scandal, like many others in recent decades, is a predictable consequence of the way the British government runs its affairs. The principle, told at length and usually with boastful pride in British high-school textbooks, is that the government should consist only of people who are members of the British Parliament and that it governs only with the consent of the Parliament or, in this context, of the House of Commons. That, the argument goes, is how it should be. All members of the government are personally answerable to one or other of the two legislative chambers, while it makes sense that the House of Commons should have the dominant role in deciding what political party shall form a government, given that it consists of people who have run for public office and who have been democratically elected. The government, like executive branches everywhere, must then administer affairs, seeking legislative authority from the Parliament whenever necessary. The British conceit is that the system of cabinet government is uniquely virtuous. The theory is that all decisions, small as well as large, are made collectively by a group of senior ministers who are the sole repositories of executive power.

There are some obvious and long-recognized defects of this system. The convention that governments may govern only with the consent of the House of Commons does not make Members of Parliament individually powerful. On the contrary, political party discipline usually ensures that they toe the party line. The result is that they are mostly impotent fellows, with very little influence even on the details of legislation, although they have during the past decade won the right to question government departments about their policies and even to complain about them. But even this right is proscribed; it will be virtually impossible for the House of Commons defence committee to get to the

bottom of the Westland affair, as it intends, if the government insists on its right not to make documents available or to allow civil servants to be questioned. But the same convention of government by parliamentary consent, coupled with the practice of fierce party discipline, means that governments are rarely changed except at general elections. This is the origin of one of the most disconcerting features of British politics since the Second World War, the sharp swings of policy that occur when one government succeeds another.

The convention that only the cabinet collectively can make decisions is similarly crippling of intelligent public discussion. Mr Heseltine's views about the manufacture of helicopters were at odds with the cabinet decision that the British government as such should have no opinion about the future of the Westland company, but this decision appears to have been reached not in a general discussion of the issue, but by a small subcommittee whose conclusion was then rubber-stamped by the rest of the cabinet. British academics and researchers, smarting from the way in which they are unable to perform their own jobs well for lack of funds, will now be asking whether the twenty-three members of the British cabinet have all deliberately decided that this is the fate the basic research enterprise in Britain deserves, or whether that too is the opinion of a few which has been given the appearance of consensus by the convention of collective responsibility.

There is worse to be said, bearing more directly on the Westland affair. The convention of collective responsibility means that only members of the cabinet can be seen to have taken decisions. Even if a minister has merely followed the recommendation of his civil servants, the convention is that the decision is his alone. Moreover, there is nothing in the British system that compels him or her to explain how the decision has been reached, while the documents preparatory to important decisions remain secret for thirty years unless the government decides they should be published. (Fair play, the present government has been more liberal, if selectively, in this respect than most of its recent predecessors.) Indeed, there is an Act of Parliament that makes it an offence for people other than ministers to release such information; it is even a criminal offence to be the recipient of such information. This is why Mr Heseltine, now an ex-minister, could make public the unpublished part of the letter he was sent by his erstwhile colleague in the government, the Solicitor-General, or a second letter that would have undone some of its damage; for that, he had to wait on the Prime Minister's pleasure, until Monday.

None of this implies that information bearing on what the government has decided or intends never finds its way into the public domain. The British newspapers are full of it. But much of the most important information is made public only by means of deliberate leaks, either by ministers or civil servants sympathetic to their cause (as with Mr Brittan's leak of the Solicitor-General's letter) or, about as commonly, by civil servants who believe their minister is wrong to (as when, at the end of 1984, Mr Clive Ponting, then a civil servant, sent anonymously to a newspaper a document prepared by Mr Heseltine about British public opinion on cruise missiles and, more recently, some civil



servants sought to dissociate themselves from Sir Keith Joseph's disastrous green paper on higher education). The manipulation of information in this way is bound in the long run to undermine public understanding of what the government is doing even if, in the short run, it renders ministers more powerful and relatively immune from criticism.

Britain's complacent tolerance of these ways of doing government business probably implies that there is little hope of change. Yet, the Westland affair is merely the most spectacular of the ways in which even governments are caught out by their own malpractices. Why should not Mr Brittan have denied receiving a letter from British Aerospace (it had been sent to the Prime Minister) when he had no reason to fear that it would be made public? And why should he not have leaked another knowing that such things go on all the time?

The less spectacular damage done to British public life by this seamy side of the British constitution is even more serious. The violent swings of policy on important issues such as education and the management of the economy have done great damage, wasting time and resources and creating a general sense of uncertainty within Britain and outside. The convention that only ministers matter is not only bad for their opinions of themselves (and their manners) but means that government policy is often no more convincing than the person who happens for the time being to be its personal embodiment. Legalized secrecy modified by vicarious and selective leaking, which may be as damaging to governments (as the past few weeks have shown) as to their electors, saps people's confidence in the process of government. And the personality traits which this poisonous combination of malpractices engenders are infectious. All the way down the hierarchy of government, there are civil servants and the chairmen of public corporations who enjoy the power of keeping secrets — and of letting some of them slip. The folly of the second part of the Official Secrets Act was acknowledged almost a decade ago (and Mrs Thatcher's predecessor promised at one stage to get rid of it). Might not she start where he gave up, and offer some such reform as expiation in the trouble she has stumbled into? □

Max-Planck survives

The 75th anniversary of the Max-Planck-Gesellschaft is a time for celebration.

THE Max-Planck-Gesellschaft is celebrating its seventy-fifth anniversary this year with a greater sense of security than would have been possible at any previous time since its formation, as the Kaiser-Wilhelm Gesellschaft, in the deceptively secure days just before the First World War. Originally the vision of the theologian Adolf Harnack (who was the society's president until 1930), the first two institutes had hardly been founded on the plot of ground donated by the Kaiser at Berlin-Dahlem before the Great War began. In what must have seemed bewilderingly quick succession, there followed the financial storm of the 1920s, the Nazi government and the Second World War. Only since the society was allowed to begin again, temporarily at Gottingen, in 1946, can it have seemed as if the stability promised by the founding fathers might yet materialize. In the event, the society was formally reconstituted, with its present name (wished on it by the British army then in occupation of Lower Saxony), only in February 1948.

Among publicly supported research organizations, the Max-Planck or MPG is distinctive and may even be unique. The original conception, that of a network of free-standing research institutes devoted largely to basic research, was not merely in 1911 quite different from anything elsewhere, but was a break with the traditions of German scholarship which, throughout the nineteenth century, had turned around the doctrine of Wilhelm von Humboldt that teaching and research are inseparable. Part of Harnack's ambition was that German science (which, in the old

tradition, covers the humanities as well) should secure a measure of independence that the German universities could not then aspire to. This was to be secured by setting up the society with a generous endowment, and 10 million marks of the day were indeed collected in the three months before the Kaiser-Wilhelm Gesellschaft came into being at 11.00 a.m. on 11 January 1911. At the outset, the society was responsible for its own running costs and capital works, although the government paid the salaries of institute directors, but the inflation of the 1920s quickly put paid to that, even though the society was still clever enough to recruit Einstein from Switzerland. Mercifully, the doctrine of independence survived, at least until the National Socialists took office in 1933.

There followed the society's most difficult period. The edict that Jews were unwelcome became the law. Fritz Haber resigned because two of his division heads had to be expelled for being Jewish. Carl Neuberg, director of the institute for biochemistry, was forced into early retirement and eventually, in 1939, forced to leave Germany altogether. Between 1933 and 1938, nearly a third of all the scientific members of the society were forced out, mostly on racial grounds. Max Planck himself, who had succeeded Harnack as president and who believed that the Nazis would disappear as quickly as they had arrived, resigned once the twenty-fifth anniversary had passed.

It says much for the strength of the network of the 1930s that it was somehow able to survive the Second World War. The British insistence that the name should be changed was natural in the circumstances. The remarkable feature of the post-war reconstitution is that nobody thought it feasible to change the constitution that, among other things, gives the members of the society the right to elect their president. The result, in the circumstances of post-war West Germany, is that the government of the Federal German Republic naturally regarded MPG as a means by which West German science could be rapidly strengthened. Especially during the 1960s and 1970s, the network of research institutes grew quickly. Now there are sixty of them, with a total strength of 10,000 people of whom 4,000 are scientists. They range in size and scope from huge research establishments such as that for plasma physics at Garching near Munich to small informal places, hardly distinguishable from university departments, such as that for mathematics at Bonn. MPG has an annual budget of DM 1,000 million, which is a mere 2.2 per cent of what West Germany spends on research each year. Its influence inside and outside West Germany is disproportionately great.

Why has the organization succeeded so well? Part of the explanation is that the institutes have indeed allowed people to get on with research, free from teaching and administrative duties. But the network has cleverly done its best to avoid the obvious danger that it will become ingrown by insisting on its right to hire people from outside West Germany, both as investigators and as advisers. MPG has also been able to insulate its people from the social upheaval in the universities that has occupied much of the past twenty years. In many ways, it has had an easier task than its publicly supported partner in research support, the Deutsche Forschungsgemeinschaft (DFG), with a budget of roughly the same size and a remit to support basic research in the universities.

Not everything is cheerful, however. Self-consciously though it has tried, MPG has not preserved all its institutes from inwardness. Now, with a static budget, the going may be harder; starting a new institute seems to require that one already in being should be closed. Moreover, the days when MPG seemed the best way to strengthen West German science have gone. The universities are stronger now, as is West German science as a whole. The obvious need is to forge better links between the universities and the sometimes still too-independent research institutes. It is good news that MPG appears to recognize the need; something like ten per cent of its scientific staff now double as university teachers. But there is still some way to go. □

Solar System

Voyager passes Uranus, Giotto rescued

Washington

VOYAGER project scientists are ecstatic about the first flood of data sent back by the Voyager 2 spacecraft during its closest encounter with Uranus.

By Monday, the total number of moons around Uranus had risen to 15, only five of which were known before the Voyager mission. A tenth ring was also discovered just inside the brightest and outermost ring around the planet, and there is evidence for ten additional faint rings. Voyager's telemetry also indicates the presence of high-energy radiation belts similar to those around Saturn, Jupiter and Earth.

The debate over the strength of Uranus' magnetic field has been resolved by Voyager. According to Dr Ellis Miner, deputy Voyager project manager at the Jet Propulsion Laboratory (JPL) in Pasadena, California, the magnetic field strength is 0.25 gauss at the cloud top. The field has a peculiar orientation, tilted 55 degrees from the rotational axis, so that one of the polar regions is pointing towards the Sun.

According to A.J. Dessler, director of the Space Science Laboratory at Marshall Space Flight Center, there is "hardly any glory for anybody" in the 0.25 gauss figure. The proponents of a strong cloud top magnetic field were expecting a figure greater than 0.6 gauss and the weak magnetic field proponents were looking for something in the range of 4×10^{-3} gauss. Dessler believes that the 0.25 figure may be revised upwards when the data from Voyager are further analysed.

Pictures sent back by Voyager 2 of Miranda, a 310-mile-wide satellite closest to the planet's surface of the five major moons, were causing wonderment among members of the photographic interpretation team.

"If you took all the bizarre geology in the Solar System and put it back into one object, that would be Miranda", Dr Laurence Soderblom of the US Geological Survey was quoted as saying.

Miranda's surface appears to be a hodgepodge of valleys, cliffs, fault zones, craters and broad terraces. The peculiar mix of features was so startling, mission scientists were loath to speculate whether external or internal forces were involved in creating the formations.

An unexpected event of Friday in the midst of the Voyager flyby gave the National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA) a chance to show how well they can cooperate.

ESA ground controllers were ex-

periencing communications problems with Giotto because of a pointing problem with one of the spacecraft's antenna systems. As it happened, a large number of the Giotto team were at JPL for meetings, and after a brief consultation, NASA turned over a portion of the Deep Space Network of tracking stations to ESA. The Giotto scientists at JPL established a direct link with Giotto mission control in Darmstadt, West Germany, and the problem was resolved within hours with no loss of data from either mission. **Joseph Palca**

Asbestos ban?

Washington

THE US Environmental Protection Agency (EPA) has succumbed to growing pressure to ban all uses of asbestos. The agency last week formally proposed a regulation that would place an immediate ban on asbestos in five use categories, and phase out all other uses within 10 years. All forms of asbestos are treated similarly by the proposed regulation.

Regulations to ban the use of asbestos, based on studies by the Consumer Product Safety Commission and the National Academy of Sciences, were first drafted two years ago. Both studies essentially concluded that no safe lower limit for exposure could be established and led EPA to decide that asbestos should be banned where substitutes are available. But the regulations were held up by objections on cost grounds from the Office of Management and Budget; EPA's administrator, Lee Thomas, said last week that the new proposal had resolved the budget difficulties.

A final decision on whether to go ahead with the ban will be taken after a 90-day comment period. A spokesman for the Asbestos Information Association said a ban would be "totally unjustified and out of step with the international consensus on current use". The association points out that the estimated 3,300–12,000 deaths each year in the United States that can be attributed to asbestos are the result of exposures of 20 years ago and more. Nevertheless, EPA estimates that the ban will save 1,000 lives before the end of the century, and officials say the ban now has a lot of momentum. The EPA proposal would ban immediately manufacturing importing and end-processing of asbestos roofing felt, flooring felts and tiles, asbestos cement pipe and fittings, and asbestos clothing. Other uses would be phased out over 10 years. **Tim Beardsley**

SDI

Software rows

Washington

THE difficulty of developing reliable software for the Strategic Defense Initiative (SDI) has surfaced once again. An SDI advisory panel had concluded that contractors working on preliminary design concepts for a space-based shield against nuclear weapons have not paid sufficient attention to the problem. The SDI organization appears to have taken the panel's criticism seriously; officials have been quoted as saying that the amount spent on software problems will be increased.

Critics of SDI hold that contractors have failed to address the issue adequately because the job simply cannot be done. The advisory panel, known as the Eastport Group, does however maintain (on the basis of qualitative argument) that reliable software can be tested and developed within the next several years.

SDI software must keep track of possibly thousands of warheads and many more decoys, coordinating multiple layers of weapons systems and sensors in real time to stop as many missiles as possible from reaching their target. Those convinced that the problem cannot be solved argue that the system cannot be tested under real conditions and that it must work first time. Analogies are made with the software failures that have often delayed space shuttle launches, for example. David Parnas, of the University of Victoria in British Columbia, resigned from the Eastport Group last year on just these grounds. Parnas now says that the group has not refuted his arguments and that he is "amazed at how little thought" has been given to his publicly stated criticisms; he accuses administrators at the SDI organization of incompetence.

One central issue is the extent to which the system functions as a single coordinated whole rather than as several functionally-autonomous subunits with built-in redundancy. A distributed system would be less vulnerable to the inevitable software errors. The Eastport Group favours a distributed system because it would make the software problem more tractable; the contractors' ideas so far, however, are based on more tightly coordinated systems.

Parnas believes the contractors chose a coordinated system because they realized SDI would not work otherwise, whereas the Eastport Group went for a distributed system because the software development would otherwise be too difficult; "they're both right". The Eastport Group says that contractors have too closely followed model system descriptions intended only for illustrative purposes that were included in the two-year-old Fletcher report on the feasibility of SDI. **Tim Beardsley**

US research costs

Government to turn screws

Washington

A long-simmering controversy over how the federal government should compensate universities for the facilities and services they provide to researchers is threatening to return to the boil, fuelled by rumours of major changes in the compensation rules by the White House Office of Management and Budget (OMB). Adding to the cauldron of controversy are two reports apparently guiding OMB in formulating new policies, one by the White House Science Council and the other by the Department of Health and Human Services. Both point to significant changes of practice in reimbursing universities and other institutions for the so-called indirect costs of research.

Indirect costs can be broken down into administrative and non-administrative categories. OMB is considering fixing administrative cost reimbursement in this fiscal year at 26 per cent of the total direct costs of research carried out under federal contracts and grants, which is the national

average at present. That figure would drop to 20 per cent in 1987. OMB is also giving serious consideration to capping the portion of administrative cost reimbursement specifically related to departmental administration at 7 per cent, a figure that would further decrease the recovery of indirect costs allowed.

Particularly upsetting to universities is the widely held belief that OMB will shortly publish sweeping changes in circular A-21, the blueprint for indirect cost reimbursement, with an extremely short comment period and without prior consultation with the universities affected.

"We are accustomed to being dealt with in a fair and reasoned way", says Janet Sweet, assistant controller responsible for indirect cost control at Stanford University. If OMB proceeds as expected, it would be a "dramatic change from the reasoned approach the government has been taking", says Sweet.

The issue of indirect costs has long been divisive for university faculties and administrations. Researchers have seen the dollar amounts of their grants grow but the money available for research shrink as an ever larger portion is devoured by indirect costs.

The Health and Human Services report (*The Impact of Indirect Costs on Research Sponsored by the Federal Government at Universities and Colleges*) takes universities to task for charging faculty salaries and other departmental overhead costs to research grants as indirect costs.

Data from 13 universities show that indirect cost payments rose from \$900 million in 1978 to \$1,700 million in 1984, an increase from 36 per cent to 45 per cent of direct cost.

According to the report, departmental administrative costs now account for a third of all indirect costs. By capping allowable departmental administrative costs at 7 per cent, the Inspector General estimates that between \$225 million and \$375 million can be saved per year.

Critics of the report argue that the 7 per cent figure is unreasonable and that departmental costs vary widely, depending on the administrative structure of a university. For some, departmental costs are insignificant, but for others departments are a major part of the total university structure.

The report also suggests that many costs now charged indirectly should more properly be charged as direct costs, a version of robbing Peter to pay Paul, according to critics.

Dr Alan Barber, vice-chancellor for research programmes at the University of California, Los Angeles, says the arbitrary assignment of 7 per cent for allowable

departmental administrative costs "makes a sham" of years of trust between universities and government auditors.

Others are not convinced that attacking administrative costs will solve the problem of rising indirect costs. Dr David Blake, associate dean for research at the Johns Hopkins School of Medicine, points to a study by Hopkins and Stanford University showing that non-administrative costs rise nearly ten times faster than administrative costs at Hopkins, but only three times faster at Stanford.

"It's clear to me", says Blake, "that all of the real increase in indirect cost has been in facilities."

The White House Science Council report (*A Renewed Partnership*. White House Science Panel on the Health of US Universities and Colleges) is less distressing for universities, although it also advocates that the growth of indirect costs should be contained by limiting administrative cost reimbursement, in this case by establishing over a two-year period a fixed level for reimbursement for indirect costs as a percentage of direct costs.

The report contains several suggestions that will clearly please universities, among which are less paperwork and, specifically, the removal of the hated "effort reporting" instituted by auditors to force faculty to say how their time is divided between administration, research and teaching. Accelerated depreciation of equipment and facilities and tax credits as a way of encouraging industry participation in capital expenditures are also suggested by the report.

But the White House Science Council's recommendations would cost money. The report says that "the source of such funding in these times of fiscal stringency is not obvious".

Joseph Palca

Panel's prescription

Washington

THE White House Science Council report, released in draft form on 17 January, raises doubts about the preparedness of US universities to face the coming decades.

The doubts are engendered both by the government and the universities, according to a special panel on the health of US universities and colleges chaired by David Packard, chairman of the board of Hewlett-Packard Company. On the one hand, the panel says, federal support has not allowed universities to meet the demands for new talent and new knowledge, while variable levels of federal support have prevented universities from making the most efficient use of their resources.

On the other hand, the report chastises universities for "attempting to ride out what were hoped to be temporary budget shortfalls" by mortgaging their research futures to "maintain research personnel rather than investing in needed instrumentation and facilities". Researchers themselves are criticized for being too conservative in their research goals.

As well as the specific recommendations relating to reimbursements, the report urges the federal government to help restore university infrastructures by forging stronger ties between industry, universities and the federal government. It also advocates that the durations of research grants should be longer and that investigators should have more flexibility in distributing grant monies and altering research goals.

Joseph Palca

Poles sentenced

The three Polish scientists from Torun accused of illegal possession and use of a television transmitter (see *Nature* 319, 170; 1986) were last week each sentenced to 18 months imprisonment while the electronics specialist in whose apartment they were arrested was sentenced to a year in prison. All sentences were suspended, however, on condition of good behaviour for three years, so that Professor Jan Hanasz and Dr Zygmunt Turlo were able to return at once to their work at the Copernicus Astronomical Centre. (The third scientist, Dr Leszek Zaleski, is a pensioner.)

Colleagues of the accused had been campaigning throughout the past four months for their release from pre-trial custody. The presence of Dr Hanasz, in particular, they explained, was essential for many important astrophysical experiments, including joint research with the Soviet Union and France.

Vera Rich

Japanese education

Uncertain future for reform

Tokyo

JAPAN's special Council on Education Reform, personally set up by the Prime Minister in 1984, delivered its draft recommendations last week. "Diversity, flexibility and decentralization" are seen as the keys to transforming the present rigid, hierarchical education system. But vigorous opposition to the plans, especially from the Teachers' Union, must put their implementation in doubt.

The 25-member council has looked at everything from kindergarten education to the support of university research. At the top, the council proposals are conventional: extension of the postdoctoral system, begun in Japan only last year with just 160 fellowships, and funds to bring more foreign researchers to Japan. It is also suggested that the minimum length of study prescribed for masters' and doctoral courses be reduced to allow talented researchers to get ahead quickly.

Reform of the universities is more controversial for it is here that the root of the education system's problems lies. It is not average standards of education that worry the Japanese; 35 per cent of all 18-year-olds already attend university and 95 per cent of children pass through senior high school. The problems are excessive uniformity and competition.

The two problems are inextricably entwined. Top universities can provide a passport to top jobs so it is little surprise that there is severe competition to enter Tokyo University, for example. What is surprising is that all Japan's universities, and even faculties, can be finely ranked according to how difficult they are to enter. That is possible only because of uniformity: the Ministry of Education closely controls school curricula and textbooks, there are selective courses at high school and university entrance examinations are of the multiple-choice type that produces scores on a single scale. That scale both defines a student's place, and by the score at which a university will accept students, a university's place, in the hierarchy.

The good side of the system is that it is completely open and fair; the bad side is that it ignores individual vocation and often tests abilities of little real use. In the English examination, for example, subtle grammatical points are tested in multiple-choice questions but not the ability to write or speak English, which would be impossible to quantify accurately.

The reforms are intended to loosen up this system. It is proposed that it should be made easier for students to transfer from one university to another through a "credit" system recognized by all universities or by a new degree-conferring body. Diversity in the universities themselves is to

be encountered by loosening the Ministry of Education's strict rules for the establishment of new universities and by granting freedom to put together new faculties without ministerial interference. The establishment of a "university council" responsible for both public and private universities that can make recommendations direct to the minister is also proposed. Further possibilities include allowing students to graduate in three years instead of four and lowering the university entrance age — at present the talented cannot move ahead of others.

These changes, although they scarcely seem revolutionary, all have their critics. One of the chief objections is that the system is indeed being redesigned to help the talented and does nothing for the great mass of ordinary students. But really fierce criticism is reserved for the proposed reforms of pre-university education.

Just like universities, high schools are graded. The ten or so schools in a typical catchment area are ranked according to how many students they send to which universities and their entrance examinations, taken on completion of junior high school, are correspondingly severe. Once entrance has been achieved, however, there is no streaming by ability nor much choice over what is studied. Thus two Japanese desires are satisfied: educational competition is free, fair and measurable while there is no discrimination among members of the same group.

The council's reforms, however, mostly centre on improving the quality of teachers by extending probation periods, reviewing teacher performance and removing those found wanting, and bringing in older and retired teachers to train newcomers. These proposals are seen by the left-wing Teachers' Union as a direct attack on the union and an attempt to increase government control over education. The union's counter-proposals are more practical: reduce class sizes to a maximum of 35 students, eliminate huge schools, increase the number of public high schools and eliminate the high-school entrance examinations.

The total opposition of the teachers makes it hard to see how reforms will ever be enacted. What will happen in the future is strongly linked to the fate of the Prime Minister, who has taken a personal interest in the reform council. Under party rules, his term of office expires in October and cannot be extended. If he retires then, little more may be heard of the education council. But there is still a chance that he will call a snap election to prove his personal popularity and then force a change in the party rules so as to stay on.

Alun Anderson

US animal drugs

FDA chastised by Congress

Washington

THE US Food and Drug Administration (FDA) has denied charges by a congressional committee that it has "consistently disregarded its responsibility" for assuring the safety of drugs used in food-producing animals. Following publication of a scathing report by the House of Representatives' Committee on Government Operations which details several failures by FDA to meet its legal obligation, FDA felt obliged to issue a statement claiming that "the US food supply is the safest in the world". But FDA admitted that the committee had found "deficiencies", many of which have already been repaired.

The government operations committee, chaired by Representative Ted Weiss (Democrat, New York), is an oversight committee: it has little power to enforce. But it has succeeded in its objective of drawing the issue to public attention.

The major charge is that 90 per cent or more of the 20,000 to 30,000 "new" animal drugs on the market have not been certified by FDA as "safe and effective", thus violating the Food, Drug and Cosmetic Act. FDA does not dispute the finding, but points out that 90 per cent of the animal drugs actually used — by volume — have in fact been approved; many of the rest are vitamins, mineral supplements and liniments that are exceedingly unlikely candidates for causing harmful effects in people. And in reply to a charge that only 7 per cent of new drugs on the market have even been catalogued, FDA says a new data-handling system should ensure that the backlog is cleared by September 1986.

Weiss's committee goes on to list "potentially unsafe" animal drugs that, it says, FDA knew presented carcinogenic risk but for which there are no adequate methods for detection of residues, as required by law: dimetridazole, ipronidazole and carbadox. And the committee quotes FDA's own investigators' conclusion that veterinary prescription drugs are widely and illegally available.

The fairest judgement seems to be that FDA's Center for Veterinary Medicine, which has responsibility for the area, lacked adequate resources to follow the letter of the law and so concentrated resources in areas where they would do most good.

While the legal failings are not easily dismissed, the extent of any actual risk to public health arising from drug residues is unclear; the extreme difficulty of extrapolating from animal carcinogenicity tests to human carcinogenesis makes attempts to quantify risk almost meaningless. But in such a cancer-conscious country, nearly is not good enough.

Tim Beardsley

British universities

Academic leaders answer back

BRITISH university vice-chancellors have made a stinging reply to the government's discussion paper on its future policy on higher education, saying that the policy document published last May is "blinkered". This strong language appears in the formal response to the policy document by the Committee of Vice-Chancellors and Principals (CVCP), the representative body of British university heads.

The document is chiefly significant for its directness, and for the bluntness of its language. On many occasions, CVCP has been able to make only a muffled response to statements of government policy because of differences of opinion among vice-chancellors, who are in any case unable to speak for their universities.

CVCP now says that last year's policy statement, technically a green (discussion) paper (see *Nature* 315, 265; 1985), "failed to address itself adequately to the problems which we face". "Nor did it display any sense of the way in which education is both necessity and catalyst in a modern society."

The CVCP response does not set out to be a full reply to all the points raised by the government, saying that its views may be taken as identical with those of the University Grants Committee (UGC) on matters not explicitly dealt with. The committee deals with the chief questions raised as follows.

Student numbers. CVCP disagrees with government projections that the numbers

of university students can be allowed to fall between now and 1995 (when the present demographic decline of the entry age-groups will begin to be reversed). Its report says flatly that there will be no decline in the demand for higher education, and points to the conflict between government statements about the importance of skill in a modern economy and its plans that the output of graduates from the universities should be reduced by 14 per cent by the end of the next decade. CVCP remarks that the proportion of people with first degrees in the British labour force was only 6 per cent in 1980, compared with 19 per cent in the United States, 13 per cent in Japan and 8 per cent in West Germany.

Tartly, CVCP also draws attention to the conflict between present policies and the green paper's own calculation of the "social rate of return" on funds spent on higher education, which concluded that the "gains from higher education overall are satisfactory even when these are limited to a purely financial calculation". If allowance were made for the benefits of personal development and other intangible consequences of higher education, CVCP says that investment in higher education might well yield returns twice as great as "from any comparable investment".

CVCP also points to the way in which the government's ambitions for an improvement in the quality of high-school education will be jeopardized by the decline in the numbers of graduates, noting that, in 1984, 30 per cent of physics teachers and 26 per cent of mathematics teachers had no qualifications in those subjects. In contrast, CVCP notes the conflict between the government's belief that the quality of school education can be improved and its assumption that this will not increase the demand for higher education.

Students' affairs. CVCP complains that the government has allowed the value of students' maintenance awards to decline by 12 per cent since the beginning of the decade, and has shifted an increasing proportion of the cost of higher education onto parents. It remarks that the government has failed to fulfill its promise to introduce a more rational system of student support, and says that if the government will not introduce an equitable system of student maintenance grants, it should devise a loan scheme instead. CVCP says that there should be more support for the European scheme to encourage the international movement of students, and that the government has "misjudged" the importance of "cultural diplomacy" in charging full fees to students from overseas.

Continuing education. The vice-chancellors accept that universities have a fuller part to play in the provision of post-experience education, but their document dissents from the government's view that the costs should mostly be borne either by industry or the individuals concerned. Noting that this policy ignores the national benefits of continuing education, the CVCP document insists that there should be at least two changes in the present arrangements; government help with the initial costs of post-experience courses and an understanding that fees paid by individuals for continuing education should be tax-deductible.

Research. CVCP notes the common misuse of the term "research" and suggests that part of the reason why technology transfer in Britain may have been laggard is the relatively small proportion of 1.6 per cent of Gross Domestic Product (GDP) spent on civil research, compared with 2 per cent in the United States and 2.5 per cent in West Germany and Japan.

The document goes on to plead for a continuation of the practice by which research councils and UGC have jointly supported academic research, the latter by means of the annual subvention of universities. On links between academic and industrial research, CVCP says the reduction of the numbers of graduate students by about 10 per cent in the past decade works against the government's ambitions that universities should do more to help industry, that the potential usefulness of universities to help British industry by the publication of new knowledge has been impeded by the relative neglect of the scientific literature by the putative beneficiaries and that the government should set up a fund to assist with the direct exploitation of academic innovations.

Salaries. The CVCP response to last year's green paper is most scathing in what it has to say about the conditions under which university teachers work. It says that declining salaries have created "grievance and distrust" whose effects, by way of recruitment, will have the effect of making universities less efficient and effective. The present age-structure of British academic staff is such that too few vacancies will arise in each of the next ten years to allow for the replacement of 3.5 per cent of the teaching force each year, even if British universities had the funds to fill all vacant posts, which seems unlikely.

In sum, CVCP conveys an air of exasperation with the government's demands on British universities. "The government asks for more and thinks that it can provide less and less without adulterating the quality of what is produced. The response to the green paper concludes by pleading that "the people of this country" should take "a political decision" about the resources to be devoted to higher education. □

SFC defies Congress

Washington

JUST weeks before its own demise, the Synthetic Fuels Corporation (SFC) has given a new lease on life to the Union Oil Shale Project in Parachute Creek, Colorado.

In its final scheduled meeting, the SFC board signed a contract for \$327 million in loan guarantees for the Parachute Creek project. The contract must still be approved by the Treasury Department before the funds will be released.

Congress voted at the end of last year to terminate SFC, and specifically prohibited it from spending any more money (see *Nature* 319, 89; 1986). SFC, however, unilaterally decided that the loan deal was not a new expenditure, but instead a formalization of a part of a larger price support agreement reached last October.

Opponents of the synthetic fuels programme are incensed by this latest move, but they admit that if the Treasury Department approves SFC's action, the government will probably be stuck with the obligation.

Joseph Palca

IVF

Another bill bites the dust

THE medical research councils of nine European countries have agreed on a policy about *in vitro* fertilization (IVF). Recognizing the need for "informed public debate", the councils last week sent British Members of Parliament (MPs) copies of a statement detailing their conclusions. The statement reached MPs in time for a proposed debate in the House of Commons in which Mr Ken Hargreaves was reintroducing a bill to ban the creation or use of human embryos except for "enabling a child to be borne by a specified woman", a re-run of Mr Enoch Powell's bill which did not succeed in the last parliamentary session. In the event, the bill was not debated because of lack of time; it is unlikely to be revived.

The nine research councils (Denmark, Finland, West Germany, Italy, Sweden, the Netherlands, United Kingdom, Austria and Belgium) agree that IVF research should be restricted to the "pre-embryo", the collection of dividing cells before the appearance of the primitive

streak, and should have the aims only of reducing infertility, of reducing congenital and hereditary disorders and of the development of safer contraceptive methods. Cloning of pre-embryos, manipulation of the genome to alter characteristics, growing pre-embryos beyond 14 days and the production of hybrids are all banned. Each country is urged to set up an ethical committee "to advise doctors and scientists and to inform and reassure the public". The committee should also contain "substantial lay membership".

The British Medical Research Council has given a £400,000 grant to the University of Edinburgh for IVF research. The programme will first develop a test to identify accurately healthy pre-embryos. The identification of gross embryo abnormalities in the laboratory will lead to a better chance of live births from replaced embryos, fewer multiple births and reduction in the risk of genetic disease, according to Professor David Baird, leader of the project.

Maxine Clarke

Academic redundancies

Committee retires in confusion

A HOUSE of Commons inquiry into the circumstances under which British academics have been prematurely pensioned off collapsed last week when it seemed to the committee concerned, the powerful Public Accounts Committee, that the National Audit Office (NAO) and the Department of Education and Science (DES) could not agree about the consequences of the rapid contraction of the university system begun in 1981.

The committee had met to consider a report on the contraction of the British university system prepared by NAO which dealt with the question whether the cost of retiring academics early, estimated at £238 million, had been a sensible use of public money. The report (HC 598) is still available from Her Majesty's Stationery Office at £2.80 even though the Public Accounts Committee, on the showing of last week, plainly considers it valueless.

The committee first looked into the effects of government policy towards the universities before the first cuts took effect in August 1981, and was then told that some 3,000 academics and 4,000 non-academic people would have to leave the universities. In the event, by September 1984, a total of 4,400 academics, but only 2,800 non-academics, had left. In retrospect, NAO estimates that the extra cost of the compensation paid to those leaving early consists of £153 million paid directly by the University Grants Committee together with an estimated £85 million

universities will have to contribute towards pensions schemes (out of their recurrent grants) in the years ahead.

Last week's fiasco seems to have arisen because the permanent secretary at DES, Sir David Hancock, drew into the discussion matters outside the period covered by the NAO review, which was exclusively concerned with the redundancy of university staff members up to September 1984. Thus he drew attention, for example, to the scheme for the appointment of "new blood" lectureships at the universities, offering this as one of the ways in which DES had helped to undo some of the damage done in the universities.

This is where the parliamentary system comes up against a brick wall. It is accepted that civil servants cannot criticize the policies of the governments for which they work, but if unpleasant things occur as a result of those policies, when does the cataloguing of those events add up to criticism?

The result, last week, was that the parliamentary committee became too confused to carry on. Believing that NAO and DES were somehow in disagreement, it sent them away with instructions to produce an agreed report. But DES said earlier this week that the existing document had been agreed between the two parties. Could last week's abortive committee hearing have been a clever ploy to postpone discussion of an embarrassing report?

Charles Wenz

US research grants

Experiment in informality

Washington

A UNIQUE experiment intended to cut down the time spent by researchers on tedious form-filling is due to start next month in Florida. Officials of five federal agencies have agreed that, for the next two years, they will use a single simplified form of research grant in the hope of reducing the administrative burdens of most principal investigators.

The Florida Demonstration Project arose out of discussions organized last summer by the National Academy of Sciences' government/university/industry research roundtable. In contrast with many similar past discussions, this led directly to a decision by officials to devise a test of how proposed improvements might work. Remarkably, the scheme has been set in place in just eight months.

The basic problem, the group agreed, is that existing federal accounting procedures tend to work best for projects with specific goals, rather than for research, which is by its nature unpredictable. Furthermore, as things are, accounting procedures are often duplicated at institutional and state levels. The end result, for the principal investigator, is that there are several forms to fill in, while Washington must be consulted in writing whenever funds allocated for foreign travel are used for buying a new piece of laboratory equipment, for example.

The new standard grant removes many of the cumbersome restrictions on normal grants, leaving the institution to which a grant is made responsible for accountability. Explicit prior approval would, for example, no longer be necessary should an institution choose to risk spending grant money before its award is confirmed.

Another irritation is the need to keep accounts from different grants separate, even when the work being supported may be hard to distinguish. Under the simplified grant, only the requirement that the scope of the research or the principal investigator should not be changed are retained. Dr Thomas Walsh of the University of Florida at Gainesville estimates that principal investigators might save up to 15 per cent of their time under the new rules.

The five research agencies that have agreed to participate in the scheme are the National Institutes of Health, the Department of Energy, the Department of Agriculture, the National Science Foundation and the Office of Naval Research. Nine institutions of higher education in Florida have agreed to be guinea pigs. Enthusiasts hope that all federal research grants in universities will eventually be modelled on the new grant.

Tim Beardsley

French nuclear power

Problems of plenty and success

NUCLEAR power provided more than two-thirds of French electricity in 1985. With six more 1,300-MW reactors coming on line in 1986, more later, and four more ordered by the government from the national constructor, Framatome, could there soon be too much? That now seems the view of Marcel Boiteux, director-general of the national utility, Electricité de France (EdF), in an astoundingly open interview with *Le Monde*, France's leading daily, earlier this month. Boiteux has thus brought into the open a festering conflict between the utility and ministry of energy about the number of nuclear reactors France actually requires.

According to Boiteux, there are two major issues. First, EdF is now in debt to the tune of FF216,000 million (£20,000 million) due to its long period of investment in nuclear construction — but the government's policy of low pricing for electricity means that EdF cannot pay off the debt (a large fraction of which is in dollars) but can only cover the interest on its borrowing.

Second, although French electricity demand grew more than 7 per cent last year, EdF's need for new power sources is slight. Framatome has been required to develop and introduce a so-called "grey mode" technology, which allows a reactor to be switched from full power production to just 30 per cent within minutes, to cope with sudden falls in demand. If EdF had had its way, there would have been only one order for a reactor in 1984 (there were two), and none in 1985–87.

But EdF's overcapacity may be relatively shortlived. Orders would have had to begin again in 1988, Boiteux said, to start replacing earlier reactors coming to the end of their life. And, apart from the debt question, nuclear power had kept French electricity prices 30–50 per cent lower than they would have been if the utility had had to rely on coal or oil-fired power stations. The reactors will also "remain profitable" even if they are run at only 30 per cent of capacity, the EdF director insisted.

Meanwhile, Framatome, which is geared up to produce not one reactor a year as is now ordered by the government to 1989, but four, will be covering the gap with a recent order for two reactors from China (though these are rumoured to have been offered at below cost), and by a new strategy of shifting heavily towards after-sales service, concentrating on robotic aids for maintenance of reactor cores and steam generators. Those services will not be limited to Framatome reactors in France, but will be offered throughout the world to all reactor owners, including those in the United States.

One area of French business is nevertheless booming — the nuclear fuel cycle, where the national nuclear fuel company, Cogema, has announced that its revamped reprocessing plan at La Hague near Cherbourg on the Channel coast has worked so well over the past year that it can now reduce reprocessing charges. Previously, La Hague had been plagued by inefficiency and shutdowns, but last year its UP II plant was able to reprocess 418 tons of spent fuel from pressurized water reactors, compared with its nominal 400 tons. Cogema plans to quadruple that capacity to 1,600 tons a year by 1992, which will further reduce costs, and claims that it is already 11 per cent cheaper to reprocess fuel through La Hague than to deal with it in any other way (such as stockpiling).

Superphénix, the world's first commer-

cial-scale fast breeder reactor, has also just delivered the first tentative fraction of its full 1,200 MW of power to the French grid at Creys-Malville in the south of France, in what has been a busy few weeks for the French nuclear industry. The reactor, which is 51 per cent French, a third Italian, and 16 per cent German, Dutch and Belgian, is functioning as expected. But according to Boiteux, there is no hope for the moment of a "series" of reactors based on the Superphénix design being profitable, or competitive with the (over-supplied) EdF pressurized water reactors. The last thing EdF would want at present is another fast breeder — as is being planned by the national nuclear agencies of Europe. But, said Boiteux, anxious not to be too much of a thorn in the nuclear flesh, it would not be unreasonable to expect fast breeders to be profitable by the end of the century — and it is certainly necessary, in the meantime, to maintain the viability of the existing fast breeder design teams.

Robert Walgate

Nuclear power

Yugoslav plans approved

THE Yugoslav federal government is now accepting tenders for the construction of two nuclear power stations by the end of the century. According to present plans, a further two will be started in the year 2000. During the past few weeks, however, there has been a wave of opposition to the whole concept of nuclear power, on both economic and safety grounds. The situation is further complicated by Yugoslavia's federal structure. The Yugoslav constitution put considerable decision-making power in the hands of the governments of the six constituent republics, but foreign currency controls mean that a major contract with the hard-currency area must be decided at federal level.

The arguments advanced for and against atomic power during the past few weeks have been fairly predictable. A typical headline in the Belgrade daily *Politika* was "100 years of risk" above a photograph of the Three Mile Island power station. Yugoslavia has coal reserves for at least 100 years, say the opponents of nuclear power, so why not at least proclaim a 25-year moratorium as the Scandinavian countries have done? Moreover, although Yugoslavia does have some uranium of its own, if this runs out, the country might become dependent on one of the major powers for fuel supplies, which would imperil its non-aligned stance. It would be far better, therefore, to devote the huge sums of money that would otherwise be spent on nuclear power stations to better exploitation of Yugoslavia's potential water-power, developing energy-saving technologies and investigating unconventional energy sources, including solar

energy and biomass. The pro-nuclear lobby, on the other hand, maintains that a nuclear power programme would create jobs in the construction sector and provide a boost for Yugoslav science and technology generally.

So far, Yugoslavia has one nuclear power station, at Krsko on the Slovene–Croatian border. A second nuclear station was proposed at the end of the 1970s, was eventually shelved due to the policy of economic "consolidation" (retrenchment) introduced at the beginning of the 1980s.

The new plans so far have proposed only one site, Prevlak, also on the Slovene–Croatian border. The Slovenes, however, are not particularly enthusiastic about another joint project. The construction of Krsko was fraught with difficulties and delays, which not only greatly increased the costs, but also caused friction with the US supplier (Westinghouse); the Slovene government began to import more and more electricity from Austria which it now considers a cost-effective alternative. Croatia has considerable energy problems, but cannot afford to construct a nuclear power station on its own. Other countries either lack the necessary infrastructure, or are unsuitable geographically. Serbia proper would be possible from the point of view of infrastructure, but so far no site in the Serbian republic has been seriously mooted. It may be significant, therefore, that during his visit to Italy last week, the Croatian premier Ante Markovic was reported to be seeking long-term credits from the Italians to help finance construction of a nuclear station.

Vera Rich

French science

New role for academy?

THE French Académie des Sciences, which began as an informal club for such *savants* as Descartes and Pascal (and was formalized by the Sun King, Louis XIV), is stuck: stuck in ancient rules which forbid it to increase its tiny membership of 130 full fellows. Compared, for example, with the Royal Society of London, with over a thousand fellows for a similar national population, the French academy is both small and powerless. Compared with the US National Academy of Sciences, it is almost non-existent, and yet there are plenty of highly motivated French scientists who believe that the academy could and should play a larger role. And this new role may be on its way, to judge by a speech made by the academy's new president, M. André Blanc-Lapierre, earlier this month.

M. Blanc-Lapierre is the energetic president of one of France's élite engineering schools, the Ivy League of French education, which provides the cream of management, the civil service and the political parties, and must be well aware of the nature of power and influence in French society. He says there has been a "certain decline" in the influence of the few reports that the academy produces and wants a rapid increase in membership.

The Académie des Sciences is one of five academies that together form the illustrious Institut National des Sciences et des Arts, which was formed from existing institutions shortly after the French Revolution of 1789. At the top of the "Institut" comes the Académie Française, with its 40 leading French writers and intellectuals — and these and the other academies of the "Institut" are not inclined to let the wayward, new-fangled Académie des Sciences actually grow, or influence government.

Under the present constitution, the only way open to increase membership would be the "infiltration" of outside talent. This, it seems, is just what M. Blanc-Lapierre has in mind. Beside the 130 full fellows, there are 80 foreign fellows and 160 "correspondents". These have no voting power in the election of fellows, but are not subject to the same restrictions in numbers as the full fellows; so Blanc-Lapierre's intention is to increase the number of correspondents by some 90 active scientists, who though unable to vote could play their full part on committees.

Blanc-Lapierre would also like to see an increase in the size of what is effectively a small academy for engineering within the Académie des Sciences, a body called CADAS (the Comité Académique des Applications de la Science) which was created in 1982. CADAS was a compromise over the establishment of a true en-

gineering academy within the Institut: The Académie des Sciences was to nurse it, inviting a membership half of outsiders (mostly engineers) and half of existing members of the Académie des Sciences. It now has nine academicians and ten outsiders but its size must be doubled, Blanc-Lapierre says, thus increasing the competence of the academy in relating science to the economy, the key political issue at present, and, incidentally, increasing the likelihood that a true engineering academy will one day be cloned from CADAS.

Whether finally the academy will really add to what is at present its principal duty, the publishing of the major French scientific journal, the *Compte Rendu* of the academy, and truly gain more influence over government through the changes foreseen may, however, be doubted. As the chairman of CODER, the Académie's Comité des Études et Rapports (reports and studies committee) said rather sadly last week, "our object has been to issue reports, but unfortunately the government hasn't asked us many questions . . . Advisory committees in France are rarely consulted". Such a situation would continue even if the academy were reformed.

Robert Walgate

Hungarian dam

HUNGARIAN opponents of the controversial Gabcikovo-Nagymaros hydroelectric project (see *Nature* 313, 615; 1985) have condemned the recent decision of the Austrian government to lend Hungary more than US\$500 million to complete the dam. The loan will be repaid in electricity once the project is completed, thereby enabling the Austrians to cancel their own plans for a similar scheme at Hainburg. This, the Hungarian statement says, is equivalent to Austria exporting its environmental problems downstream to Hungary.

However, for some years, the Hungarian government has been stalling on its part of the project — the downstream installations at Nagymaros — ostensibly due to lack of funds, but almost certainly also as a result of the increasing pressure from environmentalists. They maintain that building the three dams involved (at Dunakiliti, Gabcikovo and Nagymaros) and the diversion of the flow of the Danube through a concrete channel, will put at risk the ecology of the river, destroy the wetlands of north-west Hungary, and affect the water-table of the riparian countries, making it necessary to pipe in drinking water at a cost that will largely consume the expected economic benefit of the power produced.

Vera Rich

Marine science

Proposal for British overseer

THE proposal that British marine science should be safeguarded by a separately constituted research board is the nub of a report by the House of Lords Select Committee on Science and Technology, published this week. The committee says that such a body is needed to develop a strategy for the exploitation of the British exclusive economic zone, the 200-km offshore area that Britain has not yet formally declared. The committee says that fragmentation and the lack of funds endanger Britain's international standing.

The Natural Environment Research Council (NERC), every committee's whipping-boy these days, is criticized for its management, which the select committee describes as "the way of most resistance". Specifically, the committee says that NERC's plan that a centrally located director of marine science should superintend its own research institutes, confusing relationships between the people concerned, should be replaced by a supervisory board of which the institute directors would be members.

According to the House of Lords committee, the Marine Science Board itself should not be a part of NERC but of the Science and Engineering Research Council, although it does not follow that the research institutes should similarly be transferred.

The committee is also concerned at the budget for marine research, which amounted to £70 million for civil research in 1983–84, compared with military spending on marine research of £122 million. The committee says that two research vessels were laid up during 1985 for lack of funds (and a third was in dry dock being fitted with a new engine). Invidiously, the committee compares British spending with that in the United States and France. The Woods Hole Oceanographic Institution has a budget of \$52 million a year, while spending by the French marine research agency Ifremer exceeds the total of all British research spending from public funds in the civil sector.

The House of Lords committee also complains of the lack of coordination between the nine government departments and two research councils with a hand in British marine science. The committee argues that only a single board can be expected to take a broad strategic view in these circumstances, especially because the problems that arise are often literally global. It says that funds must also be provided by the government (although industry has, in fact, agreed to pay up to half the cost of British participation in the Ocean Drilling Project).

Peter Gambles

Free will and determinism

SIR—With reference to the letter from S.J. Starkie (*Nature* 318, 406; 1985), it is clear that freedom of will must be narrowly limited by our bodily structure and experience, but complete determinism is not credible. I can easily decide that I am so bad at guessing the interest of letters in *Nature* by reading their titles that I would do better to find a method of making a genuinely chance selection. For example, I could set up a Geiger counter in a back-ground giving an average of 20 counts a minute. Then, starting from an arbitrary time, I could record the times of occurrence of the next ten clicks. If the first occurred after 2 seconds I would read the first letter starting after 2 pages from the last Article: if the next click occurs at 7 seconds I would read the first letter starting after page 7, and so on. I would have surrendered any possible freedom of will in exchange for a programme based on a series of fundamentally indeterminate events. A newly qualified doctor could use an exactly similar method to decide in which continent he would look for his first job.

Even without such an unlikely way of making decisions, it is oversimplifying to say that either our thoughts or our actions are directly determined in any simple way by our structure and experience. Experiences are inaccurately and incompletely recorded, and the older records must have been damaged by the destruction of critical parts of cells by unpredictable fast electrons or alpha particles from randomly occurring radioactive decays from our natural radioactive background. And of

course our thoughts and decisions can be affected only by what remains of the records, not by the experiences themselves.

This is a triviality; however much damaged the records are, they could still determine our thinking.

Like most people, I *feel* that I have some freedom of will, but this feeling itself could have been programmed into me by the records of past events. While I may be programmed to think erroneously that I have some degree of free will, many of those who have thought deeply on the subject believe — or perhaps I should say are programmed to believe — that they have not. They may of course be right; but I find it difficult to accept that a rigidly determined belief in rigid determination can be a valid proof that this is how the world actually is; the argument has an undesirably circular smell.

What I am quite certain of is that we cannot get any further by talking about the problem, any more than we could have foretold the production of monoclonal antibodies by talking about heredity and immunity.

Any experimental investigation must start with a detailed study of our conscious awareness. Our consciousness is, in biological terms, very expensive. The temperature and concentrations of a great variety of substances in our brains have to be controlled far more closely than they need to be to keep the body and most of the nervous system fully operational. We should not have evolved the necessary high quality stabilization systems if consciousness were unimportant to our survival. The survival can have depended only on what we *did*, not on what we thought about what we did, so our consciousnesses cannot have been simply epiphenomena without practical influence. During the long and climatically uniform period of the Cretaceous, there could have been little advantage to an individual in diverging from the built-in instinctive behaviour that had been optimized by selection over several million years.

The sudden and permanent change of environment hypothesized to have resulted from the impact of an asteroidal body at the end of the Cretaceous period would, however, have made much of the built-in programmes inappropriate to the new conditions, and individual or group experience could often have offered a better guide to action. For this to be useful, it would have been essential to have a mechanism for deciding whether to follow the inherited programme or a new programme based on experience. I believe that our conscious awareness, useless to a creature whose behaviour was hereditarily determined, developed as the organ of choice.

When we have gained a full understanding of the way in which some ten thousand million cells, with their complex multiple interconnections, can support a single apparently unitary conscious awareness, and when we have learned how the brain so promptly finds and presents the relevant memories to the consciousness to make optimum choices possible, we can begin again seriously to discuss free will.

I expect that we shall find that our decisions are largely determined by the records of our experiences, together with a sizeable contribution from hereditarily built-in motivations, and with a random element arising from the fact that all of the records are fuzzy round the edges. When all of these components have been recognized and allowed for, we can usefully re-examine the nature, if any, of our "freedom".

J.H. FREMLIN

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SIR—Apparently one of the most deterministic aspects of human neural functioning is a compulsion to argue about free will. The recent letter by D.H. Evans (*Nature* 317, 762; 1985) claims that because the synaptic interactions among an array of neurones is far from thermodynamic equilibrium, "entropy production makes it impossible for the brain function of free will to be determined" since "causes are lost in this increase of entropy . . . as far as predicting the future is concerned". On the contrary, much behaviour is predictable despite mediation through non-equilibrium neural interactions: a host of perceptual, motor and reflex actions can be reproducibly elicited by peripheral and central stimulation.

More to the point is recognition that free will does not reflect the absence of a causal network, from increasing entropy or quantum indeterminacy of synaptic contacts or whatever. Free will implies that our volitions (a wish to argue neuroscience, to write a letter to *Nature*) causally determine certain of our actions (arguing, writing). Indeed, if such actions are not strictly determined, having these volitions would be futile. And insofar as non-equilibrium neural arrays must translate these volitions, they too must be deterministic.

The question remains, of course, as to how our volitions arise. Introspection suggests that these do not arise randomly, but that they too have causes. Free will does not imply an indeterminate or probabilistic selection, but a causally-determined choice.

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If (1986)...

SIR—

If you concede that *something* in the Cosmos
Determines every move you make on Earth,
And not resist its force but match its purpose
And tread the path laid down for you from
birth;

If you are true to Self (as Jung and Shakespeare
And sundry other schools, I think, agree)
Instead of to some norm of "right" behaviour
Or someone else you think you'd rather be;
If you will grant your favourite conviction
Will only take you far away from home
And, though it send you in the same direction,
Can only land you back where you ran from;
If you allow that, solid as you stand there,
You are no more than a character in a tale,
Of the same stuff as fantasy and dreams are,
Enacted on a universal scale,
Then you will unequivocally see
That Entropy and Order are all One,
And know how good it feels to live life free
And, which is more.....

.....you will be a god, my son.

KEITH DIXON

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Molecular biology in Tokyo

The widespread influence of molecular biology on the biological sciences was well illustrated at Nature's first conference in Japan on 20–22 January.

THOSE who think of molecular biology as a field of study, much as is botany or immunology, and that it occupies a disproportionate amount of space in many journals, this included, might have modified their views had they attended *Nature's* first conference in Japan. Held in Tokyo last week, the conference demonstrated unambiguously that molecular biology is not a subject but a tool, and one that can advance botany or immunology as well as numerous other fields of study.

To start with botany, Jim Peacock (CSIRO, Canberra) described experiments aimed at establishing the means by which certain plants respond to a lack of oxygen by inducing a set of enzymes, including alcohol dehydrogenase. A cell biologist might look to see if the oxygen-depleted cells change shape, a physiologist might measure changes in the ion concentration or pH of the cells, a biochemist would probably establish the kinetics of appearance of the new enzymes, but a molecular biologist heads for the genes. For Peacock, that meant in the first place trying to establish which DNA sequences, flanking the alcohol dehydrogenase coding sequences, are responsible for mediating the gene's induction following oxygen depletion.

The hunt, so far, has been narrowed to a stretch of about 20 base pairs, which are shared by pea and maize alcohol dehydrogenase genes. Transferred to tobacco cells, the 20-base-pair maize sequence will confer responsiveness to oxygen depletion upon tobacco but only if an 'enhancer' sequence from a gene that is active

in tobacco is also added.

It will, of course, be necessary to work "upwards" from this information to find out how the 20-base-pair stretch is activated — no doubt by a protein — how the protein is produced in response to oxygen depletion, and so forth. But at the very least, the molecular biological approach will have opened up a new way to study an old problem.

Peacock also mentioned a contribution of the molecular biological approach to plant pathology; a DNA probe for the



Professor Setsuro Ebashi

barley yellow dwarf virus can diagnose in hours the presence of the virus in the leaf sap of plants suspected of being infected by the virus, replacing a cumbersome test that took weeks or months. And for Australian farmers, molecular plant breeders hope to be able to engineer the genes of alfalfa so that a protein of high sulphur content is produced in the plant's leaves; sheep that graze on this alfalfa might yield 30 per cent more wool than at present.

Immunology

Having, in essence, solved the problem of the generation of vast numbers of antibodies from a limited number of genes, molecular immunologists have turned their attention to other phenomena. Susumu Tonegawa (Massachusetts Institute of Technology, but a graduate of Kyoto University) is concentrating on the T-cell receptor for antigen. Since it faces the same need as antibody to bind antigen, and faces the same multitude of antigens, it is not altogether surprising that it too is encoded by a limited number of genes that are able by combinatorial means to produce a much larger number of proteins. The receptor on mature T cells consists of a dimer of α and β proteins but there is also a γ chain looking for a function (to be more accurate, there is a γ gene for certain and a candidate protein). Tonegawa reported that whereas the β gene is transcribed into RNA at the same rate throughout development, the γ gene is transcribed at early stages to be replaced by transcription of the α -chain gene at a later stage. He suggested that a receptor comprising β and γ chains on the early T cell is involved in the process of learning by the cell to recognize 'self', and that the switch to an $\alpha\beta$ dimer on the mature cell enables the cell to fulfill its role of recognizing antigen in the context of 'self' molecules without being 'self' aggressive.

Tasuku Honjo (Kyoto University) and Tadatsugu Taniguchi (Osaka University) share an interest in the secreted proteins by which different cells of the immune system can speak to each other, and the cell-surface receptors for such proteins. Both have developed a system for obtaining functional receptors for one such protein, interleukin-2, from cloned DNA for the receptor, enabling structure/function studies to be started. Honjo reported that removal of most of the cytoplasmic tail of the receptor does not affect its binding capabilities for interleukin-2, and Taniguchi presented evidence that the high and low affinity receptors for interleukin-2 are products of the same gene.

Honjo's new interest is in the so-called B-cell differentiating factors, a group of ill-defined substances. Honjo's group has succeeded in cloning the DNA corresponding to B-cell growth factor 1, as defined by its behaviour in bioassays. Since the substance stimulates T cells and macrophages as well as B cells, Honjo is in-



The opening ceremony

clined to rename it interleukin-4 (a proposal that will no doubt be pondered upon by a worthy committee long after the matter has been decided by popular acclaim).

Chemistry

Richard Lerner (Scripps Clinic, La Jolla) described how molecular immunology has led him to the iconoclastic view that many peptides adopt a preferred structure in solution. His starting point was the question of why monoclonal antibodies to a short peptide corresponding to a segment of a protein can also react with the protein. At first he favoured the idea that the antigenic sites on proteins correspond to segments on the surface that have the most atomic mobility and therefore are the most likely to be able to 'match' whatever peptide structure the antibody can see. Now, however, nuclear magnetic resonance spectroscopy is providing evidence that peptides corresponding to an antigenic site on a protein adopt a structure in solution that resembles that in the



Tasuku Honjo and Hal Weintraub

protein. For example, the immunodominant peptide of the haemagglutinin of influenza virus adopts a reverse-turn conformation in solution and a peptide from myohaemerythrin has helical structure in solution.

Problems of polyglot participation

Tokyo

WHEN 'Molecular Biology Becomes Biotechnology', *Nature's* first conference in Japan, was opened by Prince Hitachi on 20 January, it became the first 'foreign' conference to be opened by a member of the Imperial family. Prince Hitachi, second son of the Emperor, is a full-time cancer researcher in Tokyo. Professor Seturo Ebashi of the National Institute for Physiological Sciences in Okazaki and Professor Toshitsugu Oda of the National Medical Centre, both of whom were on the advisory board for the conference, also participated in the formal opening.

The three-day conference, held in the Keio Plaza Intercontinental Hotel in Tokyo, was addressed by eighteen scientists from Japan, the United States, England, Switzerland and Australia. One of the speakers, Dr James Watson, had to cancel his appearance at the last minute but, with the help of two of *Nature's* staff in Washington, recorded a 20-minute video film that was shown to the audience.

More than 500 scientists attended the conference, including a sprinkling from overseas: China, the Soviet Union, Korea, Thailand, India, the United States and some European countries were all represented, but perhaps inevitably the great majority of the audience came from research laboratories in Japanese universities and industry.

Because of the inevitably high cost of mounting such a conference with many overseas speakers, the registration fee was clearly going to be beyond the reach of younger academic students. But, in the end, more than 200 of the audience were Japanese students and younger academics

whose presence was made possible by a scholarship scheme financed by 25 companies*. This was the first foreign conference to be supported by Japanese industry and also the first to be endorsed by the Japanese Ministry of International Trade and Industry.

To judge by the proportion of the audience who made use of the simultaneous translation system, at least half of them were happy to listen to presentations in English, the language used by all overseas speakers with the exception of Professor Thomas Blundell of Birkbeck College, London, who made a brief excursion into Japanese. There was much discussion among the Japanese speakers as to which language to use. In part because they felt that English is the international language of science and that all Japanese scientists need to be familiar with it, Professor Shosaku Numa and Professor Tasuku Honjo of Kyoto University, as well as Professor Tadatsugu Taniguchi of Osaka University, chose to speak in English. So too did Professor Susumu Tonegawa of Massachusetts Institute of Technology, who claimed that after many years out of Japan it was easier to cope with phrases such as 'haemopoietic stem cells' in English than in Japanese. □

*Ajinomoto Co. Inc., Asahi Chemical Industry Co. Ltd, Chugai Pharmaceutical Co. Ltd, Daiichi Sankyo Co. Ltd, Dainippon Pharmaceutical Co. Ltd, Eisai Co. Ltd, Fujisawa Pharmaceutical Co. Ltd, Kanebo Ltd, Kureha Chemical Industry Co. Ltd, Kyowa Hakko Kogyo Co. Ltd, Meiji Milk Products Co. Ltd, Mitsubishi Chemical Industries, Ltd, Nippon Kayaku Co. Ltd, Nitto Chemical Industry Co. Ltd, Sankyo Co. Ltd, Shionogi & Co. Ltd, Sumitomo Chemical Co. Ltd, Suntory, Ltd, Taisho Pharmaceutical Co. Ltd, Takeda Chemical Industries, Ltd, Teijin Ltd, Toray Industries Inc., Wakunaga Pharmaceutical Co. Ltd, Yakult Honsha Co. Ltd, Yamanouchi Pharmaceutical Co. Ltd.

Lerner also described how technical advances now allow methodical analysis of the contribution of each amino acid in a peptide to the antigenicity of the peptide. A set of peptides is made in which every one of the amino acids is in turn replaced by each of the 19 other possible amino acids, and monoclonal antibodies to each of these peptides are tested on the protein of which the original peptide is a part.

The information so obtained can pinpoint the crucial amino acid in a peptide and the essential parts of that amino acid; for example must it be charged? And that information can then be fed back into what is known about the structure of the protein to obtain, in favourable cases, a very detailed picture of what constitutes the precise antigenic site on the protein.

Hisayuki Matsuo (Miyazaki Medical



Sydney Brenner

College) has a different interest in peptides, to find and characterize new peptides from biological material. He has identified a family of neuromedins and is now pursuing the family of atrial natriuretic peptides. These peptides are secreted by the atrium of the heart and are thought to play an important role in the regulation of kidney function and blood pressure. Matsuo and others have identified three basic peptides in the family; the γ peptide is an extended form of the α , and the β is an antiparallel dimer of the α . Much effort is going into trying to establish exactly which peptides are active where, and why.

What monoclonal antibodies are to Ler-



Shosaku Numa and Hisayuki Maisuo

ner, databases are to Tom Blundell (Birkbeck College, London). Because of the problems of determining crystal structures of proteins, he is trying to derive empirical rules of three-dimensional structure on the basis of the amino-acid sequences of proteins. He is optimistic that, with the aid of computer graphics, it will be increasingly possible to recognize sequence motifs that correspond to folded structures, thus leading to improved prediction of structure from sequence.

His group's model of the structure of renin based on its amino-acid sequence and the crystal structure of homologous proteins is still being refined, partly in the interests of being able to suggest how to design better drugs to control blood pressure, but more as an acid test of the predictive techniques when the day comes that the crystal structure of renin is determined.

Development

It is clear from the events of the past year or two that molecular biology can contribute in important ways to the understanding of development. The fruitfly, *Drosophila melanogaster*, and the nematode, *Caenorhabditis elegans*, are two favoured organisms in which to carry out the studies and were accounted for in Tokyo by Wal-

ter Gehring (Biozentrum, University of Basel) and Sydney Brenner (Laboratory of Molecular Biology, Cambridge), respectively.

Gehring described some of the latest experiments on the genes that control *Drosophila* development, in particular *fushi tarazu*, which is Japanese for "not enough segments" and which is indeed a gene whose inactivation results in the loss of alternate segments in the embryo. The gene is normally expressed in stripes of activity across the embryo corresponding to the segments that form thereafter. The effects of inactivating the gene can be reversed by incorporating the cloned normal gene into the mutant embryo. And experimental manipulation of the extensive (6,000 bases) non-coding sequences upstream of the coding sequence for *fushi tarazu* has shown that separate sequences are responsible for the correct formation of stripes of activity and the pattern of activity in the nervous system.

An intriguing new result from *Caenorhabditis* is the sequence of *lin 12*, a gene that is necessary for the correct course of development. It has a sequence that is clearly related to that of the precursor of epidermal growth factor, which suggests that its product is probably involved in a cell-signalling process of some sort.

Brenner described why and how he and his colleagues have undertaken to produce a complete genetic map of *Caenorhabditis* and reported that about two-thirds of the genome has already been covered but that some of the gaps will be difficult to close.

Gene expression

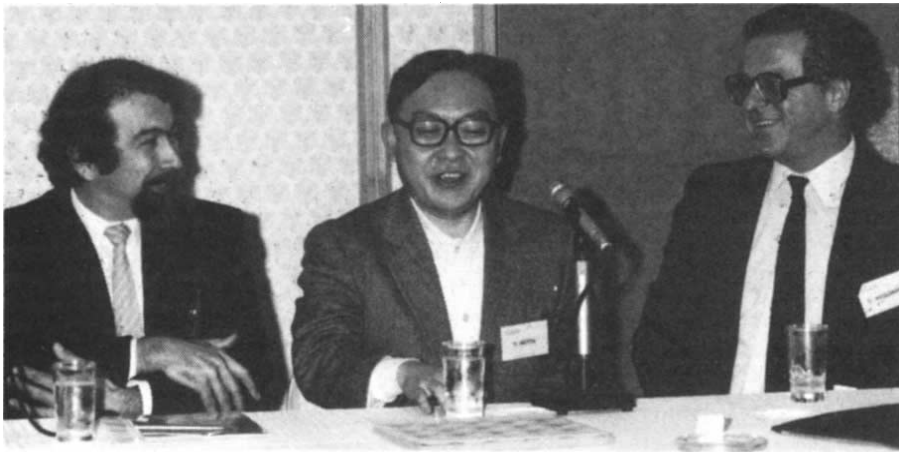
The study of gene expression is the heart of current molecular biology, or molecular genetics as this pursuit is more accurately termed. Hal Weintraub (Fred Hutchinson Cancer Center, Seattle) outlined the possibilities and problems of using 'antisense' RNA to inactivate the specific gene that is producing the corresponding 'sense RNA'. In his experience, the method can be made to work for almost any gene but it is probable that it works through more than one mechanism. Antisense RNA is likely to be an increasingly important tool in identifying the function of genes and their products.

No such help is needed by T.S. Okada (National Institute of Basic Biology, Okazaki) as his studies concern the genes for crystallin, the major protein of the lens.



His problem is to understand how the expression of the gene is normally confined to the lens when it can be experimentally expressed in other cell types. Experimental systems are proving to be a problem: only two transgenic mice have been obtained and they both expressed the crystallin gene in the brain's pyramidal neurones rather than the lens; transgenic fish were much more readily obtained but gene expression was not confined to the lens; tumours developed from teratocarcinoma cells into which a crystallin gene had been inserted expressed the gene regardless of cell type; but, more promisingly, when the teratocarcinoma cells were fused with mouse blastocysts, expression of the crystallin genes was confined to the lines of chimaeric mice that developed.

Whereas most studies of gene expression are concerned with 'front-end control', that is by the upstream noncoding sequences of a gene, Stanley Cohen (Stanford University) has concentrated on



Thomas Blundell, Yoshiki Hotta and Charles Weissmann

'back-end control', determined by the stability of messenger RNA. He described experiments that establish that mRNA (from bacteria at least) is degraded from the 3' end and that the half-life of different mRNAs can vary from 2 to 20 minutes or so. By making a variety of hybrid mRNAs between parts of molecules with very different half-lives, Cohen has shown that the 5' end of one mRNA can impose its rate of degradation upon the 3' end of another mRNA attached to it. In *Rhodospseudomonas capsulata*, he has found an example of a single mRNA (for light harvesting and reaction centre proteins involved in photosynthesis) that is degraded differentially on either side of a 'barrier', with the result that 20 times as much of one protein is produced as of the other.

Virology

One virus per speaker was the order of the day. Kenichi Matsubara (Osaka University) presented his latest data on the integra-



Question time

tion of the hepatitis B virus into the genome of human hepatoma cells. No consistent picture emerges. On the contrary, apart from the fact that in no case is the complete viral genome integrated, in no case is the same pattern of integration observed. In half the cases, the integration is complex with a variety of discontinuities and rearrangements.



The latest information to emerge from Mitsuaki Yoshida's continuing studies of the human T-cell leukaemia virus, which causes adult T-cell leukaemia, shows that the *pX* gene encodes two proteins in addition to the protein that is responsible for the transactivation of transcription.



Yoshida does not yet know the function of these two new proteins, which are encoded in a different reading frame and are mainly to be found in the nucleus.

Charles Weissmann (University of Zurich) is more concerned with the mechanism of interferon-mediated resistance to viral infection than in the viruses themselves. He has recently identified a

protein that is induced by interferon and seems to be responsible for the specific resistance of Mx^+ strains of mice to influenza virus. The protein is localized to the nucleus of cells, consistent with the large number of basic amino acids revealed by cDNA sequencing. Incorporation of the DNA into Mx^- cells confers upon them resistance to influenza virus. Weissmann suggested that resistance to the virus might therefore be transgenically conferable upon Mx^- animals but that the results might not be altogether beneficial because why otherwise should the majority of laboratory strains of mice and even a sizeable proportion of wild mice be Mx^- ?

Evolution

Walter Gilbert (Harvard University) described the technique of direct genomic sequencing and some of its applications. It enables the direct sequencing of gene fragments in a mixture of low complexity without the need first to purify the fragment. The essential requirement is enough knowledge of the sequence to make probes that are used to 'hook' the fragment.

From a knowledge of the coding sequence for triose phosphate isomerase of corn, that technique was used to identify the gene's intron/exon boundaries. A comparison of the location of the eight introns so found with the six in the chicken's gene, five in *Aspergillus* and the absence of any introns in yeast's gene, provided Gilbert with a strong argument in favour of the primitive origin of introns — that is to say, the ancestral triose phosphate isomerase gene would have contained all the introns represented in contemporary genes, and perhaps even more, and that introns have been lost in the course of evolution. That conclusion and recent evidence of certain introns with a phosphodiesterase activity led Gilbert to put forward an RNA view of the origin of life in which RNA with enzymatic and transpositional activities emerged before proteins. Proteinaceous enzymes evolved later because of their superior enzymatic function. And DNA evolved last as a means of perpetuating information.

Peter Newmark



Stanley Cohen and Shosaku Numa

Vision

Shortcomings of an eagle's eye

from Graham R. Martin

EXCEPTIONAL sensory capacity has often been invoked as an explanation of apparently unusual behavioural performance. For example, superiority of visual resolution over that of other animals is often cited to explain the hunting prowess of diurnal birds of prey: modern ornithology texts quote estimates of maximum visual resolution in the larger eagles of up to ten times that of man. However, as knowledge has increased of the physical and physiological factors that ultimately limit the visual resolution of vertebrate eyes¹, visual scientists have come to question the possibility that visual performance in these birds can attain the high levels claimed. The first evidence that visual performance in a raptorial bird does not outstrip that of man came three years ago when maximum spatial visual acuity in a small falcon, the American kestrel, *Falco sparverius*, was shown just to equal that of man². Now, the first measurement of the spatial visual acuity of an eagle's eye shows that its resolution out-performs that of man by a factor of two at best³.

Working with a wedge-tailed eagle, *Aquila audax*, Liz Reymond of the Australian National University trained the bird to learn a two-choice simultaneous discrimination task. The bird viewed the stimulus panels from six metres and expressed its choice by flying to one of two perches placed in front of the stimuli. With this arrangement it was possible to measure how the bird's visual resolution varied with both the contrast and luminance of the stimuli; Reymond was also able to test human subjects in the same apparatus to provide direct comparisons.

Reymond's findings are important since they show that the maximum performance of this eagle's eye can be predicted in a straightforward manner from knowledge of the focal length of the eye and the dimensions of the cone photoreceptors in the central fovea of the retina. Thus they provide a confident base for the prediction of visual resolution in other raptors and also demonstrate that there is no need to invoke a special role for the fovea in providing some sort of local magnification of the retinal image to enhance acuity, an idea which has been presented a number of times⁴. It may simply be that the eagle's fovea is no more than an area where the neural layers have been radially displaced so that light scattering is reduced before it reaches the cone photoreceptors.

The dimensions and packing density of the retinal cone cells in the eagle's fovea were found to be very close to the ultimate limit predicted by the waveguide

properties of photoreceptors⁵. It should be a simple matter to predict the maximum spatial resolution to be found in the eye of other eagles as long as the size of the eye, or preferably the focal length of the eye, is known. (The focal length of the wedge-tailed eagle's eye is about 22 mm; that of the human is about 17 mm.) In absolute terms the eye of the wedge-tailed eagle is among the largest known and so it is unlikely that maximum spatial resolution in any other bird eye will be greater.

Reymond's study also shows that the eagle's eye is specialized for vision at the highest light levels that occur naturally. This has definite penalties once light levels start to fall and, in the eagle, spatial resolution deteriorates more rapidly with reducing light levels than in the case of man.

Meteorites

Unexpected Antarctic chemistry

from George W. Wetherill

THE meteorites found in Antarctica during the past 16 years are clearly different from those found elsewhere in several well-understood ways. Approximately 7,000 have been collected — they are on average much smaller than other meteorites, because it is easier to identify a small black fragment on a glacier; and they have been on Earth much longer, because they have been relatively protected against weathering by being encased in glacial ice. On page 390 of this issue, J.E. Dennison and colleagues report another difference that is both surprising and less easy to explain¹. They find that the concentrations of a number of trace elements in a sample of Antarctic meteorites is different from the concentrations in a similar-sized sample of non-Antarctic meteorites. Several explanations can be suggested but various reasons make all of them difficult to believe.

The great importance of Antarctica for the recovery of meteorites was accidentally discovered by Japanese glaciologists in 1969. Since then, frequent expeditions from several nations have collected thousands of meteorites, probably representing more than 1,000 separate events. Dennison *et al.*¹ report neutron activation measurements on 23 stony meteorites from Victoria Land, near the Ross Sea and McMurdo Sound, all of the same chemical class and metamorphic grade (H5 ordinary chondrites). The analyses were compared with a sample of 20 non-

Again this is in line with predictions based on the physical factors that limit the ways in which information can be extracted from an image by an array of receptors⁶. It also explains why raptors rarely hunt at or after twilight.

Ornithologists, falconers and the casual bird watcher may have difficulty in reconciling these findings with their field observations of the hunting performance of the eagle. They might, however, find comfort in the fact that the eagle's eye seems to obey in a straightforward manner the predictions of optical physics. □

1. Barlow, H.B. *Proc. R. Soc. B* **212**, 1 (1981).
2. Hirsch, J. *Nature* **300**, 57 (1982).
3. Reymond, L. *Vision Res.* **25**, 1477 (1985).
4. Martin, G.R. in *Form and Function in Birds*, Vol.3, 331 (Academic, London, 1985).
5. Enoch, J.M. & Tobey, F.L. *Vertebrate Photoreceptor Optics* (Springer, Berlin, 1981).
6. Snyder, A.W., Laughlin, S.B. & Stavenga, D.G. *Vision Res.* **17**, 1163 (1977).

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Antarctic meteorites of the same type.

For 8 of the 13 elements studied, the differences in mean concentrations of the two samples were judged to be statistically significant. Plausible reasons are presented for believing that these differences are not laboratory artefacts or sampling bias and the authors conclude that two distinguishable populations are being sampled. They also give reasons for rejecting the hypothesis that the differences are attributable to weathering of the Antarctic samples during the approximately 3×10^5 years since they fell to Earth, so they propose that the differences must be extraterrestrial in origin. They suggest that the differences result from variation with time of the asteroidal source regions from which meteorites of this kind are derived and present a scenario in which such a change in population would result from a single burst in ejection occurring after the fall of the Antarctic meteorites. They also propose that such differences may be caused by decay of meteorite fluxes with time, caused by discrete bursts at different times before the fall of the Antarctic meteorites.

No timescale is given for this scenario — the authors note that serious difficulties arise with attempts to be more quantitative. For example, an event occurring since the fall of the Antarctic samples would result in an abundance of cosmic-ray exposure ages of less than 3×10^5 years in non-Antarctic samples, but such young

ages are rare for meteorites of this kind. Explanations based on temporal decay of flux components are equally difficult to accept. Theoretical, analytical and numerical studies by a number of authors²⁻⁴ present excellent reasons for believing that the natural timescales for decay of such populations is about 10^7 years, primarily through collisions with small debris as the meteorites pass through the asteroid belt when near their aphelion. This collision timescale is consistent with observed cosmic-ray exposure ages. Not much decay would therefore be expected in 3×10^5 years.

It might be thought that 'meteorite streams' exist, analogous to the well-recognized meteor streams of cometary origin. Coherent changes in the orbits of the stream members might lead to intersection with the orbit of the Earth of one stream at one time, and with another at a different time. This analogy is not valid, however, because these cometary streams remain in coherent orbits only for about 10^4 years. They are easily observed above the sporadic background only because these small meteoroids are removed on a timescale much shorter than that appropriate to meteorites. In agreement with this, the orbits of large meteors identified as stony meteorites that have been photographed by the Smithsonian's Prairie Network⁵ are not clustered into streams⁶. It is, therefore, difficult to believe that such rapid temporal variations in the chemical composition of the terrestrial meteorite flux are any more plausible than the alternative hypotheses that were rejected by the authors. On the other hand, if the differences are real, they must

have some explanation, possibly one that has not yet been considered. If it does turn out that some apparently well-established earlier conclusion has to be rejected, this would represent an important discovery.

How can this interesting suggestion be pursued? Probably the most fruitful approach would be to convince sceptics that the distinction reported is as real as the authors claim, thereby enlisting the considerable effort that will be required to understand it. All the differences in the mean concentrations for individual elements are within the overlap of the stated 1σ uncertainties. It is not unusual for authors to use such data as evidence against a difference. Dennison *et al.* are to be commended for not taking the easy route, as data can only be expected to reveal small but important differences when pressed to the limits. One approach that would probably satisfy many would be a hypothesis that such specific differences exist between meteorites of about 3×10^5 years old and present-day ones of this kind. This hypothesis would predict that a second investigation with a new sample of meteorites would lead to similar differences in the mean concentrations of the same elements. □

1. Dennison, J.E., Lingner, D.W. & Lipschutz, M.E. *Nature* **319**, 390 (1986).
2. Öpik, E.J. *Interplanetary Encounters* (Elsevier, Amsterdam, 1976).
3. Arnold, J.R. *Astrophys. J.* **141**, 1536 (1965).
4. Wetherill, G.W. *A. Rev. Earth planet. Sci.* **2**, 303 (1974).
5. Cepelcha, Z. & McCrosky, R.E. *J. geophys. Res.* **81**, 6257 (1976).
6. Wetherill, G.W. & ReVelle, D.O. *Icarus* **48**, 308 (1981).

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Neuropharmacology

Acetylcholine and brain cells

from David Brown

ACETYLCHOLINE has been recognized as a major neurotransmitter in the mammalian brain for more than 40 years. Our knowledge of how it works at the level of the individual brain cell is still, however, rather sparse, largely because the brain is complicated and difficult to study. Over the past few years some of the difficulties have been circumvented by the development of methods for intracellular recording from single cells in isolated slices of brain tissue maintained *in vitro*. Two papers in this issue^{1,2} illustrate how such methods can be used to provide new information about the ionic conductance changes that acetylcholine produces in brain cells, and the way these changes affect neuronal excitability. They also help to provide a clearer pharmacological definition of the receptors involved.

It is known from experiments on intact

brains that acetylcholine may either accelerate or retard the firing rate of individual neurones — that is, it may either excite or inhibit them, or sometimes it does both³. Three mechanisms for excitation have so far been identified: inhibition of a slow voltage-dependent K^+ current⁴; inhibition of a slow Ca^{2+} -activated K^+ conductance^{5,6}; and activation of a cation (presumed Na^+) conductance (T.M. Egan and R.A. North, unpublished data). Hitherto, the mechanism of inhibition has not been ascertained, although experiments on the analogous responses of peripheral ganglion cells suggested that the activation of a K^+ conductance was the probable cause. Such an effect has now been demonstrated in cells in two areas of brain studied *in vitro* — the parabrachial nucleus¹ and the nucleus reticularis of the thalamus². The latter can be directly

inhibited through this mechanism by cholinergic afferents from the brainstem reticular formation; the parabrachial neurones, on the other hand, are themselves cholinergic and so it is argued that the increase in K^+ conductance might subserve a form of auto-inhibition at their axon terminals.

One of the central points arising from these and other studies is that, in the brain, acetylcholine does not simply act as a 'turn-on / turn-off' switch to initiate or stop activity, but works more like a tuning device by modifying the frequency and pattern of cell firing. Brain cells, like heart cells, show complex electrical 'behaviour patterns', determined by the subtle interplay of various time- and voltage-dependent membrane currents. Thus, K^+ -current inhibition by acetylcholine eliminates an important component of spike frequency adaptation, thereby converting the response of the neurone from phasic to tonic firing in the face of sustained excitation^{6,7}; it also promotes burst firing⁸, by releasing the cell from the braking effect of endogenous K^+ currents. It is particularly interesting that the hyperpolarization induced by acetylcholine in the reticular neurones also modifies the firing pattern of the cells, favouring a form of burst firing². It does this by driving the membrane potential into a range where a normally inactivated transient Ca^{2+} current is primed and capable of generating a burst of spikes when activated by a brief depolarization. The old classification of acetylcholine actions into excitatory and inhibitory is clearly an oversimplification.

All of these effects of acetylcholine conform to those originally described by Dale in 1914 as 'muscarine-like' in that they are imitated by the alkaloid muscarine and are antagonized by atropine — in modern parlance, they are mediated by muscarinic receptors. This contrasts with the well-known action of acetylcholine on skeletal muscle, which is mediated by nicotinic receptors. Muscarinic receptors have been isolated and purified to homogeneity as a single protein of relative molecular mass 80,000 but, unlike the nicotinic receptor, its amino-acid sequence is not yet known. Characterization therefore depends on traditional pharmacological approaches, which have revealed subtle heterogeneity in their recognition site, at least for antagonists. Although the receptors show a constant affinity for the classical antagonists atropine and scopolamine, there is a substantial difference in the apparent affinities of receptors from different loci for the antagonist pirenzepine, which has led to a provisional sub-classification into M_1 and M_2 receptors⁹. By this criterion of pirenzepine affinity, the receptors mediating the increased K^+ conductance in brain cells conform to the M_2 type¹, whereas those inhibiting K^+ conductances probably fall into the M_1 category.

Whether this apparent correlation between receptor subtype and ionic conductance is other than coincidental remains to be determined. One facet of muscarinic action which distinguishes it from nicotinic receptor activation is that the transduction between receptor activation and membrane conductance change is less direct, probably being mediated by a linkage of the activated receptor to a GTP-binding protein (G protein)¹⁰. This may directly close or open K⁺ channels, as in the heart^{11,12}, or it may activate a polyphosphatidylinositol phosphomonoesterase or inhibit adenylate cyclase, both of which have been detected following activation of neural muscarinic receptors (see, for example, refs 13, 14), thereby indirectly changing ionic permeability of the membrane through channel phosphorylation or dephosphorylation. Given the widespread occurrence of these enzymes and

of G proteins, preservation of a rigid link between receptor subtype and ion channel might depend on selective coupling to the appropriate G protein. Further studies using reconstituted systems¹⁰ may assist in unravelling these linkages. □

1. Egan, T.M. & North, R.A. *Nature* **319**, 405 (1986).
2. McCormick, D.A. & Prince, D.A. *Nature* **319**, 402 (1986).
3. Krnjevic, K. *Physiol. Rev.* **54**, 418 (1974).
4. Krnjevic, K., Pumain, R. & Renaud, L.J. *J. Physiol., Lond.* **215**, 247 (1971).
5. Halliwell, J.V. & Adams, P.R. *Brain Res.* **250**, 71 (1982).
6. Cole, A.E. & Nicoll, R.A. *Science* **221**, 1299 (1983).
7. Brown, D.A. *Trends Neurosci.* **6**, 302 (1983).
8. Bernado, L.S. & Prince, D.A. *Brain Res.* **211**, 227 (1981).
9. Hammer, R. & Giachetti, A. *Life Sci.* **31**, 2991 (1982).
10. Haga, K. *et al.* *Nature* **316**, 731 (1985).
11. Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M. & Hillie, B. *Nature* **317**, 536 (1985).
12. Bretweiser, G.E. & Szabo, G. *Nature* **317**, 538 (1985).
13. Fisher, S.K., Klinger, P.D. & Agranoff, B.W. *J. biol. Chem.* **258**, 7358 (1983).
14. Olanos, M.C., Onali, P., Neff, N.H. & Costa, E. *Molec. Pharmacol.* **23**, 393 (1983).

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Celestial mechanics

Testing Solar System stability

from P.J. Message

THE stability of the Solar System has proved a very fruitful question for stimulating the invention of mathematical tools in the two centuries since the powerfully illuminating researches of Laplace and Lagrange¹. We now know that the successive approximations of perturbation theory indicate stability, in the framework of mutual gravitational attractions of the planets, to whatever finite order of the perturbations we press the calculations. The convergence of the infinite series thus derived is, however, not straightforward and we do not yet know the limits of validity of the expressions obtained. Consequently, tests by numerical experiments using computer integrations of the equations of motion over long spans of time are of considerable importance. The LONGSTOP project, some early results of which are reported elsewhere in this issue² has now provided an integration of the four outer major planets over 9.3 million years, revealing long-period variations in the major semi-axes of the planets' orbits. Such oscillations support the indications of long-term stability predicted by analytical perturbation theory.

The question of Solar System stability has very often been interpreted as whether the mutual gravitational attractions of the planets will leave the principal characteristics of their orbital motions unaltered. These characteristics are generally taken to be that the orbits are nearly circular, nearly coplanar, spaced well apart so that there are no very close approaches between two planets, and of approximately their present sizes. In the real Solar

System, there are also non-gravitational forces on the planets, albeit very small, some of which are dissipative in effect and will, after a very long time, ultimately disrupt the present form of the system.

The question posed for the purely gravitational system is especially challenging mathematically and the answers obtained over the past two centuries have reflected the increasing rigour and precision of mathematical techniques and understanding. Laplace and Lagrange¹ first found parameters describing the shapes and orientations of the orbits in terms of which the differential equations for the long-term changes in the orbits are approximately linear. They showed that the approximate linear equations indicate stability and the changes in the parameters are the superpositions of periodic oscillations with periods of up to about two million years, termed secular variations.

Laplace also showed that the sizes of the orbits, as measured by their major semi-axes, are subject only to periodic oscillations, to the first order in the perturbations. In an appropriate formulation, this is also true to the second order, as announced initially by Poisson^{3,4}, but, using the method generally employed in the nineteenth century to construct theories of planetary perturbations, not to the third order^{5,6}. This indicated possible underlying steady changes (secular terms) in the sizes of the orbits, suggesting a possible threat to the ultimate stability of the Solar System from gravitational interactions alone.

A series of more recent investigations, using methods originated by Poincaré⁷⁻¹⁰,

has made it clear that the variations are all periodic, if appropriate parameters and method of proceeding to successive orders of the perturbations are chosen, to whatever order of the perturbations we work. Some of the periods are very long, as already found in the theory of the secular variations. It is now clear that the secular terms in the major semi-axes shown by earlier methods are simply the start of such oscillations of very long period, appearing as power series in the time. But because of the convergence problems and the consequent difficulty in determining for what sets of parameters and time span these results are valid, stability is not yet proven by these methods.

It is clear that error bounds found by straightforward applications of the ordinary processes of mathematical analysis are much too pessimistic and grossly underestimate the true scope of validity of these expressions. Experiments by numerical integration have, however, been made in systems that do not share all of the well-ordered characteristics of the real Solar System mentioned above or in which the planetary masses have been artificially augmented. These show departures from well-ordered stable behaviour, sometimes referred to as wildness¹¹⁻¹⁴. But, to be relevant to the actual Solar System, numerical experiments must not use fictitiously augmented values of the planetary masses and instabilities are not expected to manifest themselves before the elapse of a very long time, if at all.

The integrations performed by Hubbard and Oesterwinter¹⁵ covered one million years and verified that the shape and orientation parameters showed only the superposition of long-period oscillations predicted by the secular variation theory over that interval. A more recent integration, made by Kinoshita and Nakai, covering five million years, has also led to the identification of a significant interchange of angular momentum between the orbital motions of Jupiter and Uranus with a period of just over 1.1 million years¹⁶. This is a manifestation of a beat frequency between two of the fundamental oscillations of the secular variations.

1. Laplace, P.-S. & Lagrange, J.L. *Nouv. Mém. Acad. r. Sci. Belles-Lett. Berlin* (1781); Oeuvres de J.L. Lagrange, Vol. 5, 123 (Gauthier-Villars, Paris, 1870).
2. Milani, A., Nobili, A.M., Fox, K. & Carpinio, M. *Nature* **319**, 386 (1986).
3. Poisson, S.D. *J. Éc. polytech.* **15**, 1 (1809).
4. Duriez, L. *Astr. Astrophys.* **68**, 199 (1978).
5. Haretu, S.C. thesis, Univ. Paris (1878).
6. Eginitis, M.D. *Annls Obs. Paris, Mém.* **19**, H1 (1889).
7. Poincaré, H. *Méthodes Nouvelles de la Mécanique Céleste*, Chs 9, 10, 21 (Gauthier-Villars, Paris, 1893).
8. Newcomb, S. *Smithson. Contr. Know.* No. 281 (1874).
9. Duriez, L. thesis, Univ. Sciences Techniques, Lille (1979); *Astr. Astrophys.* **54**, 93 (1977).
10. Message, P.J. *Celest. Mech.* **26**, 25 (1982).
11. Hénon, M. & Heiles, C. *Astr. J.* **69**, 73 (1964).
12. Hills, J.G. *Nature* **225**, 840 (1970).
13. Birn, J. *Astr. Astrophys.* **24**, 283 (1974).
14. Nacozy, P. *Astr. J.* **81**, 787 (1976).
15. Cohen, C.J., Hubbard, E.C. & Oesterwinter, C. *Astr. Pap. Am. Ephem. Wash.* **22**, Pt 1 (1972).
16. Milani, A. & Nobili, A.M. *Nature* **310**, 753 (1984).

Now the LONGSTOP project, the long-term gravitational study of the outer planets begun under Professor Archie Roy of Glasgow University, has covered 9.3 million years and hopes ultimately to cover up to ten times this span. Analysis of results² has shown long-period oscillations in the major semi-axes of the individual orbits, or in their energies, which are functions of the major semi-axes. In particular, they find an energy exchange between Uranus and Neptune with the same period

as that previously found between Jupiter and Uranus¹⁶. Although 9.3 million years is still a small fraction of the history of the Solar System, the LONGSTOP integrations show no evidence for gradual secular changes and thus they validate, over this period, the results of analytical perturbation theory. □

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Ocean Drilling Program

Formation of the Norwegian Sea

*from the Leg 104 shipboard scientific party**

A MAJOR objective of the Ocean Drilling Program is to increase our understanding of the formation of a deep ocean basin and its subsequent development through time. The early history of such events is hidden beneath the Earth's passive continental margins, where thick covers of sediment often make detailed studies difficult. The continental margin off Norway, however, is a relatively young geological feature, and thus is particularly well suited for studying problems of this kind. Although systematic geophysical surveys off Norway have revealed buried features on the lower continental slope that seem to be closely related to the break-up of a continent and the earliest history of sea-floor spreading, no consensus as to the nature and crustal composition of these features had been reached before ODP Leg 104

drilling of a transect across the Vøring Plateau off northern Norway.

The primary features revealed by past surveys in this region are structural highs delimiting sedimentary basins. The formation of these basins off the Norwegian coast culminated in the late Jurassic, considerably pre-dating the time of initiation of sea-floor spreading in the Norwegian Sea estimated at 56–58 Myr ago. Beneath a cover of sediment, the tops of the highs are characterized by a pronounced seismic marker of basaltic origin. Below the basalt are zones of seaward-dipping reflector sequences resting on an acoustically homogenous basement complex. Similar seismic features have been reported at many other passive margins where there is little evidence for extensional faulting before spreading.



Fig. 1 Location of ODP Leg 104 drill sites in the Norwegian Sea.

During Leg 104, eight holes were drilled at three sites. A deep drill hole (site 642) was located at the inner part of a prominent wedge of dipping reflectors on the Outer Vøring Plateau (see figures). Coring at site 642, in a water depth of 1,290 m, recovered a 914-m-thick volcanic sequence below a 315-m-thick cover of predominantly pelagic to hemipelagic Cenozoic sediments. The entire volcanic section is Eocene in age and contains 121 volcanic flows, 49 volcanoclastic sediment layers and 7 shallow-level dikes. The volcanic sequence can be divided into an upper and a lower series that are distinctive in the textural, mineralogical and structural characteristics of the flows, as well as in the compositions of interlayered volcanoclastic sediments. The upper series is both subaerial and subaqueous in origin, whereas the lower series is entirely subaqueous. Thus the seaward-dipping reflector sequence is now proven to consist of cyclic tholeiitic basalt flows and interbedded volcanoclastic sediments that form the upper volcanic series.

Coring at site 642 also penetrated the underlying rock unit, which is separated from the dipping sequence by a distinct reflector, K, in the seismic record. Reflector K seems to originate from a distinct volcanoclastic unit that defines the transition between the upper and lower volcanic series. Preliminary shipboard petrographic studies indicate a distinct boundary at this level. The proposed lithologic contact is supported by measurements of physical properties, the magnetic character of the rocks and a vertical seismic profiling experiment. We note that the volcanic series boundary is associated with a rapid decrease in both density and seismic velocity, giving rise to a large negative reflection coefficient.

Existing models proposed to account for reflector K have not gained support from Leg 104 drilling. Two significant observations are that the lower volcanic-

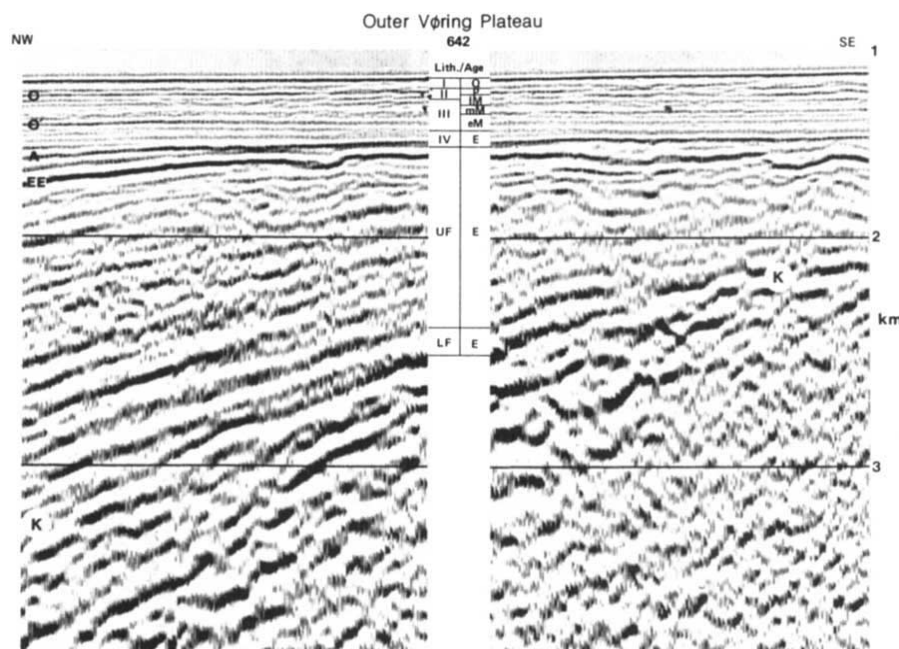


Fig. 2 Depth-migrated seismic section across the Outer Vøring Plateau, showing the sediment cover, dipping reflector sequence, reflector K and the location of Site 642. Q, Quaternary; P, Pliocene; IM, late Miocene; mM, middle Miocene; eM, early Miocene; E, Eocene; UF, upper volcanic flow series; LF, lower volcanic flow series.

flow series is apparently andesitic in nature and that interbedded volcanoclastics of the lower series contain mica, quartz and lithic fragments of continental origin. While awaiting additional chemical analyses, we tentatively suggest that the lower series is related to the transition between rift and drift phase, whereas the upper series was emplaced later, probably at the time of the North Atlantic Thulean volcanic surge in the lower Eocene (approximately 53–56 Myr ago).

A palaeoenvironmental drill-site transect combining site 642 with site 643 near the foot of the Voring Plateau and site 644 in the inner Voring Basin was used to study the evolution and variability of the Norwegian current. This current, a continuation of the Gulf Stream, transports temperate North Atlantic water along the Norwegian margin to the Arctic Ocean and provides northwestern Europe with warm climates. The Voring Plateau is well suited to study this palaeoceanographic problem because of its regular blanket of pelagic and hemipelagic sediments. The transect recovered material from environments varying from the deep ocean basin to the central continental slope, and will allow detailed studies of horizontal and vertical temporal gradients associated with the current system. The two innermost sites document only the Neogene and Quaternary history of the ocean basin (the last 24 Myr) but the sedimentary record of site 643 extends back into the early Eocene (about 53 Myr).

All sites yielded sediment sections with high-resolution Neogene and Quaternary magnetostratigraphic and biostratigraphic records, allowing detailed reconstructions of the palaeoceanography of the Norwegian–Greenland Sea and of northern hemisphere palaeoclimate. A temperate Norwegian current was long-lived during the Neogene but began to deteriorate at 2.8–2.9 Myr (possibly as early as 4.5 Myr), being replaced by intermittent cold ice-covered polar water masses. Layers of dark, carbonate-poor glacial muds, interbedded with light-coloured interglacial sediments, constitute the most conspicuous evidence that glacial climates existed in the Norwegian Sea during the past 3 Myr. At that same time, cold-water planktonic faunas and floras begin to appear in the surface water masses. In the mid-Quaternary (0.73 Myr), the intermittently occurring high abundances of biogenic opal disappear from the sediments, suggesting a dramatic drop in surface water productivity. Calcareous nanoplankton and planktonic foraminifers,

however, persist in abundance until 0.4 Myr, producing enough particles to form calcareous muds and marly oozes interbedded with the glacial layers. Around 0.4–0.5 Myr the Norwegian Sea depositional environments become almost exclusively dominated by the glacial mode.

The transition from the pre-glacial to glacial palaeoenvironment occurs 0.5 Myr

earlier in the Norwegian Sea than in the North Atlantic Ocean to the south of the Greenland–Scotland Ridge, but it is preceded by the 4–6 Myr glacial record of the Arctic Ocean. We also note that the cessation of pre-glacial conditions occurred almost simultaneously with a similar major northwards expansion of Antarctic polar water. □

Prehistoric ecology

Clues from the pollen record

from Peter D. Moore

FOR many years palaeobotanists and archaeologists have used pollen analyses of peats and lake sediments as a guide to the use of land by prehistoric communities. Certain characteristic pollen types and particular changes in pollen frequency are now accepted as evidence for the involvement of prehistoric man in the modification of vegetation. But many of these conventional interpretations have developed mainly as a consequence of informed speculation; there has been very little empirical analysis of the man–vegetation–pollen relationship. The International Geological Correlation Programme has set up a working group to consider the value of ‘anthropogenic indicators’ in pollen diagrams; the first meeting was in Wilhelmshaven, West Germany, on 3–8 October 1985.

Some of the most significant research has been carried out by K.-E. Behre (Wilhelmshaven), whose group has been excavating Iron Age and later settlements in the marshes and sandy heaths of north Germany. In the low-lying lands around the mouth of the Weser River, the waterlogged soils are valuable to archaeobotanists because of the excellent preservation of the remains of wooden artefacts, fragments of textiles and crop plants. In Iron Age and Roman times, settlements were established on *Wurten*, artificially constructed mounds of earth that elevated their populations above the level of the floods. The soils for cultivation (plaggen soils) had to be imported from the higher sandy ground; the remains of these soils and the Celtic fields of Iron Age times are still abundant in the area.

Well-documented, extensively excavated archaeological remains in close proximity to wet, peat-forming habitats, both on the ill-drained plains and in kettle-holes within the sandy uplands, have permitted close comparison of the historical and prehistorical records with the evidence from stratified pollen in the peats. In particular, the frequency of peat deposits allows the study of lateral spatial variation on the registration of cultivation events in pollen diagrams. There are also bogs of different sizes, allowing local and

regional events to be differentiated.

Kettle-hole bogs in the sandy areas are particularly interesting because cultivation took place in the immediate catchment of their basins. The site of Schwienkuhle, for instance, now lies within dense beech forest, parts of which still have the distinctive marks of an Iron Age field system. The bog is only about 30 metres in diameter, so is likely to contain a record of events that were mainly local. It is in a site like this, therefore, that one might expect to find the most marked development of anthropogenic-indicator pollen. This has proved to be the case — high pollen levels of cultivated plants such as rye (*Secale cereale*) are found, together with their associated weed flora, particularly the cornflower (*Centaurea cyanus*) which seems to be strongly associated with winter cereals. Also found are indirect indicators, such as the sorrel (*Rumex acetosella*) and heather (*Calluna vulgaris*), which owe their presence to the general disturbance of the sandy soils and the opening up of woodland canopies. During the Middle Ages cultivation reached its peak, with rye attaining 55 per cent (expressed as a percentage of tree pollen), corn spurrey (*Spergula arvensis*) 36 per cent and ribwort plantain (*Plantago lanceolata*) 60 per cent.

But one of the most interesting features of Behre's analyses from this site is not a maximum, but a ‘settlement gap’ in the cereal and weed record, which occurs in the Iron Age/Roman cultivation episodes at about AD 500 and may have lasted for as long as 200 years. This gap has posed a fresh problem — identifying the cause of the decline. So far, theories include plague and a mass emigration of population, perhaps to the British Isles.

Another area in which some progress is being made is the detection of associations between supposed anthropogenic indicators in pollen diagrams. Can one detect specific weed or crop assemblages and interpret them precisely in terms of the land use? J. Turner (Durham, UK) has tackled this question by using multivariate analyses on several published pollen diagrams from different parts of Europe. Using

*Co-chief scientists, O. Eldholm (Univ. Oslo) and J. Thiede (Universität Kiel, FRG); ODP staff representatives, E. Taylor (Texas A&M Univ.), C. Barton, U. Bleil, K. Björklund, P. Ciesielski, A. Despratras, D. Donally, C. Froget, R. Goll, R. Heinrich, E. Jansen, L. Krisek, K. Kvenvolden, A. LeHuray, D. Love, P. Lysne, T. McDonald, P. Mudie, L. Osterman, L. Parson, J. Phillips, A. Pittenger, G. Ovale, G. Schoenharting and L. Viereck.

principal components analysis (PCA) she looked for species clusters and found that some pollen types do tend to behave in similar ways.

In one site in East Anglia, for example, wheat (*Triticum*) is closely associated with Compositae (Tubuliflorae) pollen types at certain levels — perhaps the latter were the weed flora of the wheat crop. At the same site, *Secale* is found close to flax (*Linum usitatissimum*) on the PCA plots. It is unlikely that the two crops were grown together but it is possible that they were grown sequentially in a form of rotation. Not surprisingly, patterns vary in different parts of Europe. The strong correlation between rye and cornflower in northern Germany, for example, is not seen in pollen diagrams from Czechoslovakia, whereas in Central Europe cornflowers seem to be associated with buckwheat (*Fagopyrum esculentum*) in the fossil pollen record.

In the far north of Europe, the whole problem of detecting the past influences of man is compounded by the general paucity of the flora. In the region of

Bergen, Norway, P.E. Kaland has concentrated largely on the history of heathland, where it is possible to detect the effects of such land-use practices as burning, grazing and scything for fodder. The northern limit of heathland in Norway coincides with the 0°C isotherm for the coldest month — under colder conditions animals cannot be kept outside in winter, hence heathland is never developed.

Even further north, in the Tromsø region, K.-D. Vorren has met with more problems. Here, the effects of grazing encourage plants such as *Botrychium*, *Polemonium* and *Trollius*, natural components of the Arctic grassland. No longer can one rely on the standard anthropogenic indicators of the south; subtle variations in the proportions of certain meadow species must be sought. The Arctic may prove as enigmatic as the arid zone in the quest for pollen indicators of man's former activities. □

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Physics of liquids

Are diameters rectilinear?

from J.S. Rowlinson

FORMULATED a hundred years ago¹, the law of rectilinear diameters has become one of the classic laws of the physics of liquids. In the past fifteen years, however, doubts have been raised about the validity of the 'law' on several occasions. The latest and most serious challenge comes from S. Jünger, B. Knuth and F. Hensel².

The law describes the change with temperature of the mean density of a liquid, ρ_l , and of the vapour in equilibrium with it, ρ_g . The law states that $\bar{\rho} = \frac{1}{2}(\rho_l + \rho_g)$ is a linear function of temperature, T . Since both ρ_l and ρ_g approach the limiting density, ρ_c , at the gas-liquid critical point, the law can be written

$$\bar{\rho} - \rho_c = D_1 \tau \quad (1)$$

where $\tau = (T_c - T)/T_c$ and D_1 is a constant. Each density separately approaches ρ_c as τ^β , where the index β is about 0.32 (see

figure)

$$\rho_l - \rho_c = B_0 \tau^\beta; \rho_g - \rho_c = -B_0 \tau^\beta \quad (2)$$

Is the law true? Experiments seemed to confirm it. But serious doubts were first raised when it was found to be false for a theoretical model of a liquid³, which had instead the expansion

$$\bar{\rho} - \rho_c = D_0 \tau^{1-\alpha} + D_1 \tau + \dots \quad (3)$$

where the index $\alpha = 0.11$ is that which describes the well-known singularity in the heat capacity, C_v . A term in $\tau^{0.89}$ might be difficult to distinguish experimentally from one in τ , but interest was excited by the possible existence of a singularity in a function that had been thought to be well-behaved and the theoretical and experimental hunt was on.

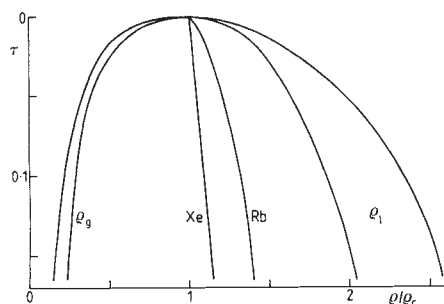
A second model⁴ was soon found that also has a singular diameter, although this result was complicated by the fact that here $\alpha = 1/2$ and $\beta = 1/4$, so that $1 - \alpha = 2\beta$, and a term in $\tau^{2\beta}$ is known to designate the 'wrong' choice of order parameter. Suppose ρ_l and ρ_g conform strictly to equations (1) and (2) but, to distinguish liquid from vapour, we choose instead a variable that is an analytic function of ρ , for example the molar volume ρ^{-1} . It would follow that for the mean of this function there is a term in $\tau^{2\beta}$ on the right-hand side of equation (1) or (3) which is an even stronger singularity than that in $\tau^{1-\alpha}$ because, for real liquids, 2β is about 0.65. There is no *a priori* reason why ρ should be chosen, but it certainly seems to be the best function in

practice as, with this choice, no term in $\tau^{2\beta}$ has ever been detected.

There were soon other theoretical models^{5,6} for which it could be shown rigorously that there was a term in $\tau^{1-\alpha}$, but the size of the coefficient D_0 was unknown. Belief in the existence of this term was strengthened by arguments based on the renormalization group^{7,8}. Meanwhile, despite much effort, the experimentalists failed to find convincing evidence: the figure shows the results for xenon⁹. The only simple liquid for which there is good evidence¹⁰ of a singularity is SF_6 , but even here it has been suggested¹¹ that the measured density of the vapour might be too high because of a wetting layer on the inside of the cell.

The whole subject has been re-opened with some difficulty, but apparently accurate, measurements of the liquid and vapour densities of the two alkali metals, rubidium and caesium, by Hensel and his colleagues at Marburg². Their results for rubidium are included in the figure and the contrast with those for xenon is clear; near T_c , $\bar{\rho}$ is not a linear function of T . Their analysis suggests that a term in $\tau^{2\beta}$, if present, is at least 30 times smaller than that in $\tau^{1-\alpha}$ for $10^{-1} > \tau > 10^{-3}$. Their values of $1 - \alpha$ are 0.86 for Rb and 0.87 for Cs, which are close to the value of 0.89 found for simple liquids from the singularity in the heat capacity.

If the long-predicted singularity has at last been unambiguously detected it is important to know why it is seen only for liquid metals and not for conventional liquids. Liquid metals conduct electricity but their vapours do not. The metal-insulator transition is at a density about that of the critical point, so Goldstein and Ashcroft¹² have argued that it is the strong dependence on density, near ρ_c , of the screened-ion interaction that is responsible for the strength of the singular term. This argument looks convincing and leads to the interesting suggestion that a similar effect might be found in the coexistence curves of metal-ammonia solutions. □



The reduced densities of gaseous and liquid xenon (inner curves) and rubidium (outer curves). Central lines are the mean densities, $\bar{\rho}$.

1. Cailletet, L. & Mathias, E. *C. r. hebdo. Séanc. Acad. Sci., Paris*, **102**, 1202, (1886); **104**, 1563 (1887).
2. Jünger, S., Knuth, B. & Hensel, F. *Phys. Rev. Lett.* **55**, 2160 (1985).
3. Widom, B. & Rowlinson, J.S. *J. chem. Phys.* **52**, 1670 (1970).
4. Hemmer, P.C. & Stell, G. *Phys. Rev. Lett.* **24**, 1284 (1970).
5. Mermin, N.D. *Phys. Rev. Lett.* **26**, 169 (1971); **26**, 169, 957 (1971).
6. Mulholland, G.W., Zollweg, J.A. & Levelt Sengers, J.M.H. *J. chem. Phys.* **62**, 2535 (1975).
7. Nicoll, J.F. *Phys. Rev. A* **24**, 2203 (1981).
8. Nicoll, J.F. & Zia, R.K.P. *Phys. Rev. B* **23**, 6157 (1981).
9. Cornfeld, A.B. & Carr, H.Y. *Phys. Rev. Lett.* **29**, 28 (1972).
10. Weiner, J., Langley, K.H. & Ford, N.C. *Phys. Rev. Lett.* **32**, 879 (1974).
11. Moldover, M.R. & Gammon, R.W. *J. chem. Phys.* **80**, 528 (1984).
12. Goldstein, R.E. & Ashcroft, N.W. *Phys. Rev. Lett.* **55**, 2164 (1985).

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Thunder in the air

Gwyn Macfarlane

The Life of Ernst Chain: Penicillin and Beyond. By Ronald W. Clark. Weidenfeld & Nicolson: 1985. Pp.217. £14.95. To be published in the United States by St Martin's Press at the end of March, \$25.

ERNST Chain (1906–1979), the third man in the trio of penicillin Nobel Laureates, has a distinguished biographer in Ronald Clark. Much has been written about Fleming, less about Florey and — apart from E.P. Abraham's admirable Biographical Memoir for the Royal Society — virtually nothing about Chain. This, the first full-length biography, is a factual account of a gifted, highly temperamental and provocative scientist.

Clark devotes only six pages to Chain's early life, and we miss the personal details that might have explained the moulding of his extraordinary character. His parents were Jewish, his father a Russian chemist who had established a chemical works in Berlin, his mother German. The death of the father and the failure of his business left the family impoverished, but Ernst's education at school and university did not suffer. He loved music, becoming a fine concert pianist and music critic for a Berlin newspaper. A career in music was considered, but he also loved science, graduating in chemistry and physiology and then studying chemical pathology at the Charité Hospital. He was, we are told, liable to "violent outbursts with shouting and throwing things about".

Under this excitability there was a native shrewdness and, alarmed by the growing antisemitism in Germany, he left in April 1933 to seek refuge in England. In London he was befriended by J.B.S. Haldane who found a post for him with C.R. Harington at University College Hospital Medical School. It was an unhappy arrangement. Chain complained about the lack of equipment, quarrelled with Harington and left within a few months "after a row". Haldane rescued him and settled him with Gowland Hopkins in Cambridge. Here he was happy, finding Hopkins "one of the most considerate and kindest of human beings". Working for a PhD, he studied enzyme chemistry and showed that the neurotoxin of a snake venom was an enzyme that destroyed a component of the respiratory chain, thus explaining (for the first time, he claimed) the biochemical action of a natural toxin.

In 1935, Howard Florey, newly

appointed professor of pathology in Oxford, was looking for a young biochemist. Hopkins recommended Chain, and Florey appointed him. For the first few years all was well between them, and Chain's work went brilliantly. Florey had asked him to study lysozyme, the natural bacteriolytic agent discovered by Fleming in 1921. Chain, with L.A. Epstein, showed that it was an enzyme and identified its substrate as a component of



Ernst Chain: "It seems to be my destiny to be involved in fights. . .".

bacterial cell walls, thus explaining its lytic action. He then proposed a joint investigation, with Florey, of other natural antibacterial agents, including penicillin, discovered by Fleming in 1928.

Clark gives a lucid and impartial account of the now-familiar Oxford penicillin story. Chain had his own version with considerable differences in emphasis, like a photographic print and its negative. Clark has achieved a synthesis by quoting Chain with qualifications. For example, Chain used to assert that it was he who introduced Florey to penicillin after reading Fleming's original paper. But Clark quotes an earlier conversation in which Florey described penicillin and the failure of Raistrick — a famous biochemist — to obtain a stable extract. "He can't have been such a very good chemist" remarked Chain. But it was, it seems, Chain's enthusiasm that set practical work in motion. The biochemical problem challenged him, and Florey's interest was in the action of penicillin on virulent staphylococci.

Chain and Epstein started work in 1938 and Clark quotes the latter as saying that "results were unimpressive". Then N.G.

Heatley joined in, and his innovative mechanical genius increased the production of crude penicillin so that Chain was able to obtain active extracts. He asked Florey to test the toxicity of these, and was annoyed when he delayed. So Chain asked J.M. Barnes to inject a mouse, and when this seemed none the worse, claimed exultantly that "this was the crucial day in the whole development of penicillin". As Clark points out, the non-toxicity of penicillin had, in fact, already been shown (by Fleming). However, Chain's impatience stimulated a rather aggrieved Florey to take active control of the whole project.

The really crucial day was 25 May 1940, when the first animal protection test proved the immense therapeutic possibilities of penicillin. The story then moves on two fronts, the drama of the first clinical trials in man and the biochemical triumphs

of Abraham and Chain in purifying penicillin and discovering its β -lactam structure. But, as success mounted, personal rifts opened between Chain and Florey. One concerned patents. Chain, the son of an industrial chemist, knew their importance, and urged protection for the Oxford processes. He was over-ruled by Edward Mellanby, of the Medical Research Council, who found the very idea unethical, and by Florey. Bitter arguments ensued and Chain's resentment was increased when, a few years later, events proved him to have been, in principle, right.

The second breach was more personally wounding. In 1941, it was agreed with Mellanby that Florey should go to the United States to seek commercial help in producing penicillin. Chain had expected to go with him, but Florey decided to take Heatley, the production expert, and the first that Chain knew of this was the sight of the packed bags in Florey's room. "I left the room silently", he wrote years later, "but shattered by the experience of this underhand trick and act of bad faith". Thereafter, as the fame of penicillin grew, Chain came to fear that he would be cheated of the credit he believed to be due to him. In 1944, premature rumours that a Nobel prize would be awarded to Fleming and possibly Florey increased Chain's agitation and he considered pressing his own claim. In fact, in 1945, the prize was equally divided between Fleming, Florey and himself.

A Nobel prize and international renown only increased Chain's sense of grievance in Oxford. He considered his colleagues' account of the penicillin work for a joint book on antibiotics "a travesty of the true facts", and his own 19 pages of amend-

From The Life of Ernst Chain

ments seriously delayed publication. In particular, he objected to the concept of an Oxford team led by Florey. He was frustrated, too, by the rejection of his proposals for a fermentation plant in Oxford, and a national penicillin factory. He began to establish outside links, becoming a consultant to various pharmaceutical firms and foreign government agencies, and to the Weizmann Institute in Israel, where a tempting position was on offer. Finally, in 1948, he agreed to direct the new biochemistry department of the Istituto Superiore di Sanità in Rome, where Marotta, the head of the Institute, could satisfy his demands for space and funds.

Chain's new department produced important work, the most significant arising from the idea of modifying the penicillin molecule to produce more effective compounds. He was a consultant to the Beecham Group, who took up the idea, sending biochemists to work with him in Rome. They isolated the nucleus of penicillin and, on returning to England, produced semi-synthetic derivatives of great therapeutic (and commercial) value. In 1964, trouble struck the Rome Institute. In an incomprehensible political vendetta, Marotta and several staff members were charged with the alleged misuse of government funds, and sentenced to long terms of imprisonment. Chain was not on trial, though the prosecution claimed that the

Italian government had a right to the Beecham patents. Meanwhile, he had left Rome to become professor of biochemistry at Imperial College, London.

Chain's plans for his (second) new department were so grand that the cost was far beyond the available College or University funds. But he refused to modify them, and himself raised over £600,000 from various sources, including the Wolfson Foundation and Lord Rank. The new buildings housed a fermentation plant, spacious laboratories and a luxurious penthouse apartment for the professor and his family. As in Rome, Chain's years there were fruitful and happy up to a stormy finale. A knighthood in 1969 had removed a growing sense of injustice. Then, in 1973, when he was due to retire, he insisted on the right to nominate his successor under the terms of the Rank gift. He was over-ruled and then fought legal battles with great tenacity but with ultimate defeat and much bitterness.

Chain's remaining years were filled by ceaseless activity. He retained many commercial consultancies and travelled incessantly, lecturing in any one of his five fluent languages, lobbying for his pet causes or against those he disliked. He was seldom for long either in his Wimbledon home, or his house in the West of Ireland, where he died after a heart attack in 1979.

Ronald Clark has produced a readable, lucid but unduly deferential book on a difficult subject. For technical matters he has evidently (and wisely) drawn heavily on Abraham's Memoir. While he faithfully recounts the facts of Chain's highly coloured career and achievements, and quotes extensively from his writings, he makes little attempt to analyse motives and character. In consequence, the book seems remote and bloodless. It is difficult, perhaps, to present a clear picture of someone in constant — almost frantic — movement, and whose violent emotional drives could change course almost overnight.

There were steadier drifts in Chain's life. Between youth and age he moved from the political left to the right, and became more firmly attached to his Jewish faith. The sense of personal injustice was a constant companion, unresolved by great scientific and material success. He never learned that too much shouting for what you want makes people deaf and then hostile. "It seems to be my destiny to be involved in fights throughout my career" he wrote (to Sir Robert Robinson) during the Imperial College affair. In truth, his destiny was simply to be himself. □

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All about being an amphibian

Tim Halliday

Biology of Amphibians. By William E. Duellman and Linda Trueb. McGraw-Hill: 1985. Pp. 670. \$40, £40.

AMPHIBIANS are of increasing importance in several areas of biology, including ethology, physiology and embryology, and a new textbook that presents an up-to-date and interdisciplinary account of the group is extremely welcome. The last such textbook was G.K. Noble's classic *Biology of the Amphibia*, published in 1931. This is, inevitably, a very different book. Apart from being much larger, it is much more densely packed with the detailed and quantitative information that is nowadays considered essential in scientific texts. Sadly, the chatty and discursive style used by Noble is no longer appropriate in such a book.

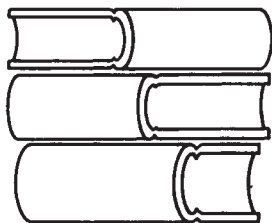
Duellman and Trueb intend that their book should be used either as an advanced course textbook or as a general reference book on all aspects of amphibian biology. They have deliberately adopted a functional and evolutionary approach, dividing the 19 chapters into four parts which deal with life history, ecology, morphology and evolution. They have relied heavily on review articles, rather than primary research papers; even so, the bibliography contains some 2,500 references, of which about a third have been published since 1980.

Individual chapters deal with topics that have a biological coherence and do not necessarily reflect divisions between academic disciplines. Thus, behaviour and locomotion are not treated as separate topics but are discussed as and when they are relevant in chapters on, for example, mating and courtship, feeding and defence. This is a particular strength of the book as it allows the biology of the animals themselves to emerge more clearly than it otherwise would.

The balance of subject matter is also good. Most fields of amphibian research are covered in some detail so that, with its bibliography, the book provides an excellent starting point for anyone wishing to study a particular aspect in detail. One area that receives less than adequate coverage is endocrinology, despite the fact that it has been the subject of a lot of recent research. It is surprising, for example, to find no mention of the hormone prolactin, given its importance in the control of fluid balance in a group of animals whose lives are so dependent on water. In selecting their examples, Duellman and Trueb set out to include animals from all parts of the world. They have been more

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successful in this than in providing a geographically comprehensive coverage of the literature to amphibian research — the references to the work of continental Europeans, particularly the Italians, are very sparse.

Perhaps the most valuable part of the book is that dealing with evolution and taxonomy. Since Noble's day, many new species have been described and the classification of amphibians has undergone radical changes. Duellman and Trueb draw on data from a variety of sources, including cytogenetics, molecular biology and biogeography, to present a coherent

and up-to-date account of amphibian phylogeny.

The text is clear and concise and richly illustrated with diagrams and numerous photographs, though some of these are too indistinct to be very informative. Amphibians and their fascinating and varied habits have long deserved a major textbook. This book goes some way towards being all one could wish for and is likely to be an important source of reference for several years. □

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A sense of the past

Patrick Rabbitt

Neuropsychology of Memory. Edited by Larry R. Squire and Nelson Butters. *Gulford Press*: 1985. Pp.655. \$75, £62.50.

AT FIRST sight this is yet another bulky, and startlingly expensive, compendium of articles. It consists of 54 review and experimental papers, loosely organized under three headings: "Studies of Normal and Abnormal Memory in Humans", "Studies of Memory in Non-Human Primates" and "Studies of Memory in Non-primates: Physiology, Pharmacology and Behaviour". On closer reading one finds that it is brilliantly edited. The contributors have not merely been selected for eminence, but because they have something important to say — and they have been disciplined to say it very concisely. In total, however, the book reads like a collection of reports by members of an irreconcilable committee, but this perhaps is the most honest and useful reflection of our current knowledge of the nature and pathology of memory.

Mayes and Meudell capture some of the main tensions in their insightful and critical review. Experts disagree whether "amnesia" is a unitary, or a multiple condition; as to which precise locus of damage to a human or animal brain produces the condition; and as to precisely what disabilities distinguish the "amnesic syndrome" from non-pathological "poor memory" and whether this syndrome is produced by damage to a single critical area or varies with idiosyncratic patterns of multiple lesions. But possibly the most important contribution of this book is that it forces us to recognize an even more fundamental weakness in memory studies: the functional descriptive framework in which all theories have been cast, all experiments designed and all results interpreted over the past 30 years is becoming increasingly unsatisfactory, but no useful alternative has yet been worked out.

This tacit framework has been that "in-

formation" from sense organs is "filtered" by a process known as "selective attention". It is then (or later) "encoded" and then (or later) "stored" as an "engram" which is later "retrieved" when required. Studies of memory have attempted to reveal the characteristics of each of these three hypothetical "information processing stages". A tape recorder can be an apt metaphor for this framework as Rose points out in his review, but he goes on further to describe its weakness.

A good example of this weakness is to be found in the paper by Hall and Loftus who consider the case where people experience a particular event and then, subsequently, receive further information which bears upon it and which, apparently, makes them misreport the original details in good faith. The "tape and tape recorder" metaphor rigidly restricts alternative explanations to "overwriting", but the main trouble with this simple underlying model is that it is hard to disprove in specific cases. Even results discrepant from Loftus's, such as those of Bowers and Bekerian, can be re-cast in the same context.

To escape the pervasive assumptions of this framework we must consider how inadequate they are to interpret the more subtle phenomena of memory loss. For example Cermak, greatly extending observations by Kinsbourne, points out that early "memories" produced by amnesics have the characteristic of inferential reconstructions. When pressed, a patient may admit that he cannot recollect any specific episode in his life when he went sailing, but can nevertheless well recall the nature of the sport and, from other incidental evidence, can specify a period of his life when he frequently sailed. Thus the interesting thing about memory is that "retrieval" of information is not simple "reading" of passive "records" by a "tape head", but rather a process of inferential reconstruction — at least as complex as that which occurs in the perceptual interpretation of sensory events. Why has the Lockian associationist fallacy, so long dismissed from perception, persistently lingered in memory studies?

It has been inevitable that rival theories of amnesia should also have been proposed in terms of loci of deficit within this three-stage model. Since it is logically impossible to demonstrate that a given piece of information is *not* held, albeit incommunicado, in "memory store", the trichotomy becomes a dichotomy — disturbances of encoding of relevant information (e.g. Butters and Cermak) or disturbances of "automatic" encoding (e.g. Hirst) as against disturbances of retrieval processes (e.g. Weiskrantz and Warrington). Once again, the model carries restrictive conviction, and even encourages very useful applied work — such as Mathis and Korner's demonstration, in this book, that, like normal people, amnesic patients are helped to learn word lists by a variety of mnemonic techniques.

Thus, though the book will be very helpful to all trying to find their bearings in a complex literature, perhaps its main contribution is to force on us the embarrassment of recognizing that we know that the models of memory we still use are inadequate, but that we do not know how to do better. Perhaps one way out of this is to try and define clearly what *kind* of model we now need. Perhaps, to shake us loose from our restrictive assumptions, we need a few Zen koans to meditate? In his excellent initial chapter, Craik, who gets nearer to the heart of these issues than any commentators before him, seems to attempt just this useful provocation:

if memory is like perceiving, then it is perhaps no more likely that memory traces exist in the absence of remembering than it is that percepts exist in the absence of perceiving . . .

and

Good memory for an event is associated with situations in which the event is related to the subject's past knowledge in a meaningful way, yet is also treated in a way that is distinctive from this past knowledge . . .

and

students of memory should not be striving to model the system "at rest" or in isolation — that is, as some entity with an existence separate from its activities. Rather our goal should be to model the interactions themselves — the interactions among tasks, environmental events, and mental representations of past knowledge. Sensai! □

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New in paperback

- *The Great Mental Calculators: The Psychology, Methods, and Lives of Calculating Prodigies, Past and Present* by Steven B. Smith. Publisher is Columbia University Press, price is \$15. For review see *Nature* 310, 79 (1984).
- *Glimpsing an Invisible Universe: The Emergence of X-ray Astronomy* by Richard F. Hirsh. Publisher is Cambridge University Press, price is £8.95, \$17.95. For review see *Nature* 306, 508 (1983).

Putting the protists in their place

F.E.G. Cox

An Illustrated Guide to the Protozoa. Edited by John J. Lee, Seymour H. Hutner and Eugene C. Bovee. *Society of Protozoologists, PO Box 368, Lawrence, Kansas 66044, USA: 1985. Pp.629. \$80 (non-members of the Society), \$60 (members).*

ONLY after a long gestation period has this mammoth publication finally appeared. It comes in large format, printed on glossy paper, and is illustrated with over 3,000 line drawings, photographs, and transmission and scanning electron micrographs. The stated objective was to produce a book "which would enable scientists and students working with protists to place them in an up to date taxonomic scheme". Certainly, every protozoologist should have a copy (or access to one) but whether it will be used as a continual source of reference is debatable.

In general, the 1980 Society of Protozoologists classification (the Levine scheme) has been used, with minor modifications to the phyla Sarcinomastigophora, Labyrinthomorpha, Apicomplexa and Myxozoa, major changes to the Microspora and a completely new classification of the Ciliophora. In other respects, the 23 authors have adopted different approaches. For some groups, particularly among the Phytomastigophorea, the reader is taken to species, in others, for example the Ciliophora, the genus, and for some, including the Foraminifera, the family. For the Ciliophora, there is an overall dichotomous key that leads to the order followed by subsequent keys to family. Other groups have keys starting with higher taxa and some, notably the Apicomplexa, have none.

Who is this book for? Certainly not the novice. I had difficulty in following some of the keys and had to resort to the well-tried technique of finding a suitable picture and working backwards. The editors point out that the original literature will have to be consulted, and most protozoologists concerned with taxonomic problems will do this automatically. But those who are not experts, or do not have access to library facilities, might be tempted to use the book as a substitute for the original literature.

Parasitologists, too, may find it a bit disappointing. Those interested in trypsinosomes will be alarmed to read on p.9 that *Trypanosoma gambiense* is a parasite of rodents, and on p.142 that the flagellum does not arise from a flagellar pocket (thus distorting the whole of the key), and will be dismayed by the diagram of "Trypano-

soma congolense" (p.151) which is actually *Cryptobia vaginalis*. Unfortunately, this cannot be checked because the reference given is not actually cited in full and is in fact wrong. Other such errors limit the book's value considerably.

Overall, this guide represents a heroic effort which does not quite come off; in retrospect, a series of specialist keys, which could be revised easily, might have been better. Treated with caution, however, this is a nice book to have around and we shall never see its like again. □

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Threads of insight

John J. O'Reilly

Fiber Optics: Technology and Applications. By Stewart D. Personick. *Plenum:1985. Pp.257. \$45, £38.03.*

STEWART Personick has been a leading figure in optical fibre research for some 15 years, and his early papers on receiver design for digital fibre optic systems are still the key references in this area. A new work by such an eminent author is thus well deserving of special attention. The book is an outgrowth of an introductory course on the subject and, consequently, the author does not go into much detail. Rather, he presents first, in Part I, an overview of components and subsystems with a view to providing a basis upon which the reader can build an understanding of the practical applications and limitations surveyed in Part II.

In addition to the usual material on principles, sources and detectors, Part I includes helpful discussions of practical transmitter drive circuits, receiver design and optical components. System impairments arising from interactions between the source and the fibre, such as modal

noise, mode partition, modal distortion and laser noise induced by reflections, are also outlined.

The applications considered in Part II range widely, encompassing telecommunications trunk transmission systems, data links, local area networks, and analogue and broadband networks. Sensing systems are covered briefly, as are system measurements. There is, too, a very brief account of emerging technologies such as integrated optoelectronics, coherent systems and optical switching.

The book is well provided with diagrams and photographs which should enhance its usefulness for the novice. In some instances, though, the reproduction process has resulted in an unfortunate lack of detail (and on one or two occasions the author refers to the colours in monochrome photographs!). The text is largely non-mathematical in nature and there is little in the way of fundamental analysis. For all that, this is not a lightweight book; what it lacks in rigour it makes up for in practical insight and experience.

The descriptions are clear, concise and simple, enabling the underlying principles to be readily appreciated, while references are provided to enable the reader to follow up on the details. The book does not, however, offer us anything new; indeed, several sections of the early chapters seem to have much in common with the author's previous book, *Optical Fiber Transmission Systems*, which was published in 1981 as a volume in the same series.

All in all, this is an interesting collection of topics, pitched at a level which will allow a newcomer to the subject to get to grips with the basic ideas and gain some insight into industrial practice. But, for me, it lacks the style and flair which characterize so much of Dr Personick's output in the technical journals. □

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Fechner's colours: as the eye scans across the diagram, the spatial pattern is translated into a temporal pattern of stimulation. Because the different cones and chromatic channels respond at different rates, unsaturated colours may be seen. The illustration is taken from a new textbook published by Harper & Row, Seeing the Light: Optics in Nature, Photography, Color, Vision, and Holography, by David Falk, Dieter Brill and David Stork. Price is \$35.50, £31.95.

The fight for science textbooks

from Thomas H. Jukes

Many scientists have deplored the success of creationists in expunging evolution from school science textbooks. A sustained and arduous effort is necessary for a reversal of this process.

CALIFORNIA in the past year has been the scene of a sustained struggle between creationists and science. For those involved, it was necessary to work with committees of educators who use a specialized language unfamiliar to scientists, with the State Board of Education, with textbook publishers and with the media. Our best results were with the media.

Led by State Superintendent of Public Instruction Bill Honig, the California State Board of Education ("the board") had started a programme to improve the quality of education. Among other things, the State Department of Education seeks to improve school science textbooks. These have long been inadequate, especially in their treatment of evolution. Genetics is described forthrightly without being termed, let us say, "Beliefs of the possibility of inheritance", but the word "evolution" is usually not mentioned for fear of reprisals by creationists^{1,2}. As a consequence, the meagre and muted descriptions of evolution in such textbooks appear under chapter headings such as "Changes over Time", or "Evidence of Change".

A Curriculum Commission, reporting to the board, supervises the steps needed for official adoption of the school science textbooks. The first step in the current round, on 24 July 1985, was a hearing, in Costa Mesa, California, on 7th and 8th grade science textbooks, conducted by the commission. Several scientists, including William Bennetta, John A. Moore and Kevin Padian, urged more and better treatment of evolution. The usual litany of objections was presented by a chorus of creationists. Following the hearings, the commission recommended that none of the books be adopted until they were revised to comply with an official publication, the *Science Framework Addendum* of 1984³. Revisions, said the commission, were needed in treatment of four topics: cell biology, genetics and evolution; earth science; human reproduction; ethical considerations. Inclusion of the magic word "evolution" alerted veterans of the perennial controversy.

On 12 September, the board met at the headquarters of the Department of Education, Sacramento, to consider the rejection of the books by the commission. Again, several scientists spoke, also three clergymen, urging revision of the textbooks, and again a number of creationists

opposed this. Next day, the board voted unanimously for revision of the books as recommended by the commission.

During the hearings, a board member, Angie Papadakis, said she was not convinced about evolution because she could not imagine that primitive creatures could have given rise to males and females, but she was in favour of science because it had produced cosmetics and emollients.

In the next few weeks, deliberations took place involving the commission's Science Subject Matter Committee (SSMC) and the textbook publishers. The chairman of both the commission and SSMC is Robert Douglas, a professional educator who is director of special services for Plumas School District, Quincy, California. SSMC had no biologist as a member, and did not seek advice from any professional biologist. (Two scientists were on the committee that prepared the 1978 Framework and wrote Appendix A.) SSMC stated on 31 October that haste was needed because revisions had to be presented to the board by 13 December for a vote on adoption of the revised books. SSMC met again on 13 November, and set a deadline of 21 November for submission of revisions by publishers. (The books and their publishers were: Heath *Life Science* and *Earth Science*; Holt *Science*; Macmillan *Life Science*, *Earth Science* and *Physical Science*; Merrill *Focus on Life Science* and *Principles of Science*; Prentice-Hall *General Science* and *Life Science*; Scott Foreman *Life Science*.)

This left twelve working days for public inspection and comment on revisions. Only one copy of the revisions was made available for inspection, because of a weird excuse that they had not been copyrighted, therefore no copying of revisions would be permitted. Viewing would be permitted only in Sacramento.

Copies of the revisions were obtained by Bennetta, however. They were perfunctory and inadequate; and in some cases actually weakened the original text. For example, the phrase "still holds true", in the sentence "Although . . . [Lamarck's] idea that animals that have homologous structures are closely related still holds true" was changed to "is still accepted by scientists". Many obviously erroneous statements remained unrevised and uncorrected. Such qualifying phrases as "some scientists think" were widely used in descriptions of evolutionary pro-

cesses, though not for other scientific phenomena. Obviously, these servilities were intended to appease creationists.

In our criticisms, we had to avoid antagonizing members of the board, or they might have second thoughts about improving the books. Also, four positions on the board would soon be scheduled for replacements, to be appointed by Governor Deukmejian. We were in a dilemma: we wanted to support Bill Honig's intent to improve the teaching of evolution, but the books were just as bad as before revision. One book said that "some scientists believe" that dinosaurs roamed the Earth millions of years ago.

Nevertheless, we could not condone the impending travesty, and Bill Bennetta lambasted the books and revisions in a widely distributed 19-page letter (2 December). This produced responses, quoting his criticisms, by newspapers, radio and television. We converged on Sacramento for the public hearings on 12 December to learn that individual statements would be limited to 3 minutes.

Douglas presented his committee report containing the clause "The antidogmatism guidelines were considered throughout this revision process" but not mentioning the fact that these guidelines refer to "discussing origins of life and Earth", not to evolution. He recommended adoption, without change, of all revisions dealing with evolution.

About 20 pro-evolution speakers then made statements, including two Roman Catholic priests, a rabbi, various biologists on the faculties of the University of California, Berkeley, San Jose State University, San Diego State University, and scientists from the California Academy of Sciences. Each of these speakers severely criticized a single textbook and its revisions in detail, and furnished a copy of his or her statement (unabbreviated to the 3-minute limit) to each board member at the hearing. However, the vote of the committee took place during the hearings, so the copies of our statements were not perused by its members.

Kelly Segraves and his mother Nell⁴, who are creationists, made their usual protest about violation of the rights of Christian children by the teaching of evolution, violations of the antidogmatism policy, and so on.

The chairman, Mr Romero, then called on Douglas for a summary, which was

mainly a rebuttal of pro-evolution statements. Douglas said he knew of no biology textbook at this level (7th and 8th grades) that used evolution as the central theme. He said our objection to the incessant caveat "some scientists believe . . ." should be ignored, because *National Geographic* magazine used this phrase in November 1985, and this august publication "is not subjected to pressures". He said that, in deference to us, he had persuaded Merrill to remove a paragraph recommending readers to arrange for a pupil to discuss creationism in science class, and that he had persuaded Heath to change "many scientists think that dinosaurs were the ancestors of modern reptiles" to "scientists classify dinosaurs as the ancestors of modern reptiles". This announcement was greeted with guffaws by the audience. Superintendent Bill Honig said that the publishers had complied with the request for more examples of evolution, including fossils, blood comparisons and description of embryos.

Discussion by board members followed. Board member Angie Papadakis said she could not understand why scientists claimed that the North and South poles had changed places several times. Why couldn't scientists make up their minds? Board member John Ward said that he had "suddenly become aware [*sic*] that large numbers of people believe in divine creation, that probably there is something other than evolution", and that

we should "leave a window where students could have some belief other than evolution" to account for their existence, "perhaps divine creation".

The committee voted unanimously to approve the Douglas report. Next day, the full board voted 7 to 2 to accept the report.

The "Antidogmatism Policy", The Science Framework for 1978 (6), Appendix A, included statements on evolution including:

"The process [evolution] has been going on so long that it has produced all the groups and kinds of plants and animals now living as well as others that have become extinct. . . . Another characteristic of life is change in its genetic material with passage of time. This process, termed evolution. . . . indicates that all living organisms on Earth have a common ancestor from which they have diverged by evolution during about three billion years. . . ."

These statements were contested in court by Segraves *et al.* in 1981¹. Judge Irving Perluss refused to change them and "found, that in discussing evolution, Appendix A of the Science Framework, along with an antidogmatism policy adopted by the State Board of Education in 1972, fully accommodates plaintiffs' religious views". The "antidogmatism policy" refers only to "discussing origins of life and Earth in public schools"³.

The *Science Framework Addendum* (1984) seems to have lost track of Appendix A, and has diluted and abbreviated the 1978 Framework's description of evolution. The Addendum assures its readers

that "all science materials [emphasis added] on the State Board of Education's approved list have been measured against the Board's antidogmatism policy. . . ." These warnings evidently cowed publishers, and inhibited them in revising their books, or even, in the case of Prentice-Hall, led to weakening of the original text.

We were permitted one small postlude. The board said that a final check of the books for factual errors, but not for method of presentation, could be made within the next week. Four of us went again to Sacramento, and gave lists of such errors to the Department of Education, and we were told that our lists would be sent to the publishers.

None of the books that we saw describes evolution as well as *The Young Scientist Book of EVOLUTION* (yes, in huge block capitals), 32 pages (Usborne Publishing Ltd, 20 Garrick St, London WC2E 9BJ). But, after all, England is the home of evolution.

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1. Jukes, T.H. *Perspect. Biol. Med.* **25**, 207 (1982).
2. Bennetta, W. *Pacific Discovery* (California Academy of Sciences, Golden Gate Park, San Francisco, California), January–March 1985.
3. *Science Framework Addendum for California Public Schools* (California State Department of Education, Sacramento, 1984).

ARTICLES

Isolation of a cDNA clone coding for a possible neural nicotinic acetylcholine receptor α -subunit

Jim Boulter, Karen Evans, Dan Goldman, Gary Martin,
Doug Treco, Steve Heinemann & Jim Patrick

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We have isolated a complementary DNA clone containing sequences homologous to those encoding the α -subunit of a mouse muscle nicotinic acetylcholine receptor. Based on the structural similarities between the encoded protein and the muscle acetylcholine receptor α -subunit, and the presence of hybridizing RNA species in the brain, we propose that this clone codes for a neural nicotinic acetylcholine receptor α -subunit.

NICOTINIC acetylcholine receptors are present at the vertebrate neuromuscular junction, in vertebrate sympathetic ganglia and in the vertebrate central nervous system. These receptors can be distinguished on the basis of the spectrum of ligands that open or block ligand-induced opening of the ion channel. For example, the elapid α -neurotoxins that block activation of nicotinic acetylcholine receptors at the neuromuscular junction fail to block the activation of nicotinic acetylcholine receptors found on several different cell types including PC12 (a chromaffin-cell-derived rat cell line)¹, chick sympathetic ganglia², chromaffin cells³ and Renshaw cells⁴. It seems likely,

however, that the nicotinic acetylcholine receptors at the neuromuscular junction are structurally related to the neural nicotinic acetylcholine receptors because antibodies directed against the muscle type receptor will block ligand-induced activation of an α -neurotoxin-resistant acetylcholine receptor⁵. To gain access to this different class of acetylcholine receptors, we used a complementary DNA clone coding for the mouse muscle acetylcholine receptor α -subunit to find, by low-stringency DNA/DNA hybridization, related sequences expressed in the PC12 cell line. We describe here the isolation and properties of a cDNA clone encoding a protein homologous to the muscle

Fig. 1 a, Partial restriction endonuclease map and nucleotide sequencing strategy for the λ PCA48 cDNA clone. Relevant restriction endonuclease sites are identified by numbers in parentheses which correspond to the 5'-terminal nucleotide in the recognition sequence. The bracketed *EcoRI* sites at the ends of the clone were inserted as linkers during the construction of the cDNA library. The extent and direction of sequence determinations are indicated by horizontal arrows. In some cases, synthetic oligonucleotide primers were used instead of the M13 universal primer for sequence determinations and these are designated by letters in brackets:

[A], 3'-AGGGGAGGGACAGATAGCCC-5';

[B], 3'-CAGTGAAGAGGGGTGTCAG-5';

and

[C], 3'-GTCACCTTCTCCCCACAGTC-5'.

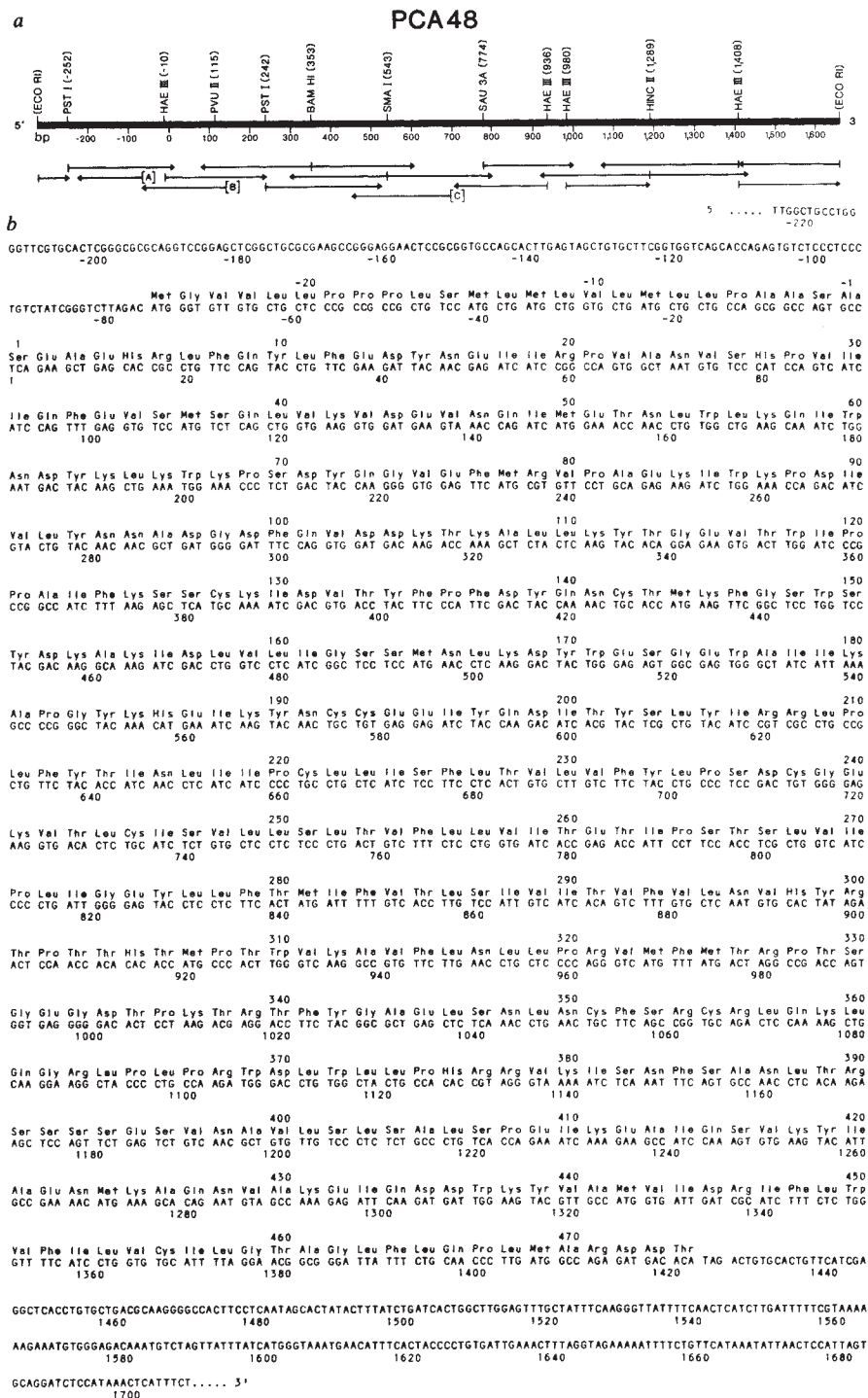
b, The nucleotide and deduced amino-acid sequence of clone λ PCA48. The nucleotides are numbered in the 5' to 3' direction, starting with the first nucleotide in the codon corresponding to the putative amino-terminal residue in the mature protein. Nucleotides extending 5' of base 1 are designated with negative numbers and include residues encoding the putative signal peptide and the 5'-untranslated region. The 5'-untranslated nucleotide sequence presented does not extend to the end of the cDNA in λ PCA48; approximately 110 nucleotides from the *EcoRI* linker at the 5'-terminus of the cloned DNA extending 3' to nucleotide -225 have been omitted. Nucleotide sequence data in this region show it to be a cloning artefact (see text); furthermore, S₁ nuclease experiments demonstrate that PC12 mRNA will only protect 5'-untranslated sequences up to approximately residue -225 (see Fig. 3). There are 281 nucleotides in the 3'-untranslated region. The λ PCA48 sequence includes residues -225 to 1,655 (the clone ends here with an *EcoRI* linker). The sequence in this region is extended 3' with clone λ PCA44, which contributes nucleotides 1,656-1,707. The deduced amino-acid sequence for the λ PCA48 clone is shown above the nucleotide sequence, with the first amino acid representing the putative amino terminus of the mature peptide.

Methods. A cDNA library was constructed using poly(A)⁺ RNA isolated from the rat phaeochromocytoma cell line PC12⁶. Size-fractionated double-stranded cDNA was prepared using the method of Gubler and Hoffman⁸, ligated to phosphorylated *EcoRI* linkers (10-mer, 5'-CCGAATTCGG-3') and cloned into the *EcoRI* site of bacteriophage λ gt10 (ref. 9). The library base was 5×10^6 plaques. Approximately 1×10^6 plaques were screened with a radiolabelled 540-bp *PvuII*/*HincII* fragment derived from the plasmid pMAR α 15 which contains coding sequences for the mouse muscle acetylcholine receptor α -subunit¹⁷. This cDNA fragment (nucleotides 132-672) codes for a part of the amino-terminal extracytoplasmic portion of the mature α -subunit as well as the putative acetylcholine binding site and the beginning of the first membrane-spanning region (amino acids 37-216). Three independent clones were isolated, λ PCA44, λ PCA48 and λ PCA54. Partial restriction endonuclease mapping (data not shown) revealed common fragment lengths for the three clones. The longest clone, λ PCA48, was ~2 kb and was selected for sequence analysis. DNA sequencing was performed using the dideoxynucleotide chain-termination method of Sanger¹⁰ and M13 bacteriophage vectors mp18 and mp19 (ref. 11).

receptor α -subunit. Based on this homology and on the distribution of homologous RNA species in the rat brain, we propose that the protein encoded by this cDNA clone is an α -subunit of a neural nicotinic acetylcholine receptor.

Isolation and sequencing

The rat phaeochromocytoma cell line PC12⁶ expresses a neural nicotinic acetylcholine receptor that is pharmacologically



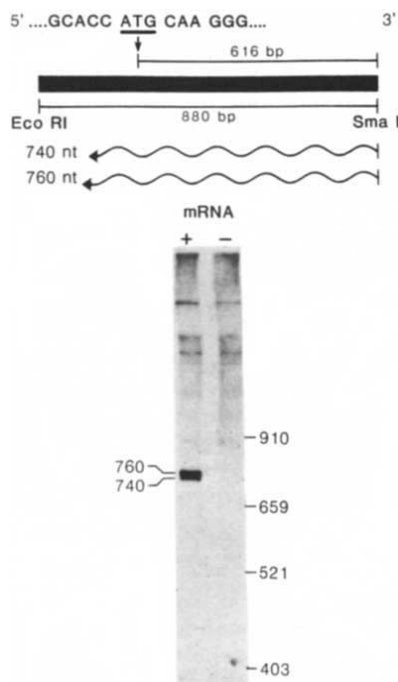


Fig. 2 S_1 nuclease analysis of the 5' end of the λ PCA48 cDNA clone, performed as described previously¹⁶. Appropriate single-stranded M13 phage harbouring the 5' *Eco*RI (nucleotide 1) to *Sma*I (nucleotide 880) fragment from λ PCA48 was annealed with or without 10 μ g poly(A)⁺ RNA purified from PC12. The sample was digested with 400 units ml^{-1} S_1 nuclease for 40 min at 40 °C, extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol and resuspended in formamide. Samples were electrophoresed on a 3% acrylamide, 8 M urea gel and electroblotted to Gene Screen Plus. The blot was probed using the nick-translated *Eco*RI/*Sma*I fragment from λ PCA48 with prehybridization, hybridization, washing and autoradiography conditions as described earlier¹⁶. Exposure time was 16 h at -70 °C. The numbers to the right of the figure are the number of nucleotides in relative molecular mass (M_r) standards electrophoresed in adjacent lanes; the estimated sizes of the two protected fragments, 740 and 760 nucleotides, are shown on the left of the figure.

The 2-kb species was always present in poly(A)⁺ RNA prepared from PC12, but the 3.5-kb species was detected only occasionally. Hybridization was not detected under conditions of higher stringency (0.2 $\times$ SSPE, 65 °C), suggesting that the hybridizing species were homologous but not identical to the cDNA coding for the muscle nicotinic receptor α -subunit.

We prepared a cDNA library using as template poly(A)⁺ RNA isolated from PC12 cells. Double-stranded cDNA was made using a modification of the Gubler and Hoffman procedure⁸ and ligated into λ gt10 (ref. 9) using *Eco*RI linkers. The library had a base of 5×10^6 and was screened at low stringency (5 $\times$ SSPE, 50 °C) without amplification using a probe prepared from pMAR α 15. This screening revealed three positive overlapping clones out of the 10^6 examined. One of these positive clones, λ PCA48, was chosen for further study.

The nucleotide sequence of λ PCA48 was determined using the dideoxy-sequencing reactions developed by Sanger¹⁰ in conjunction with the M13 strains developed by Messing¹¹. λ PCA48 was subcloned into the vector pSP65 (ref. 12) and the locations of various restriction endonuclease sites were determined. Purified restriction fragments were subcloned into M13mp18 and M13mp19 and their sequences determined. Regions not covered by restriction fragments were sequenced using oligonucleotide primers corresponding to portions of the sequence already determined. A partial restriction map of λ PCA48 and a description of the sequencing strategy are shown in Fig. 1a.

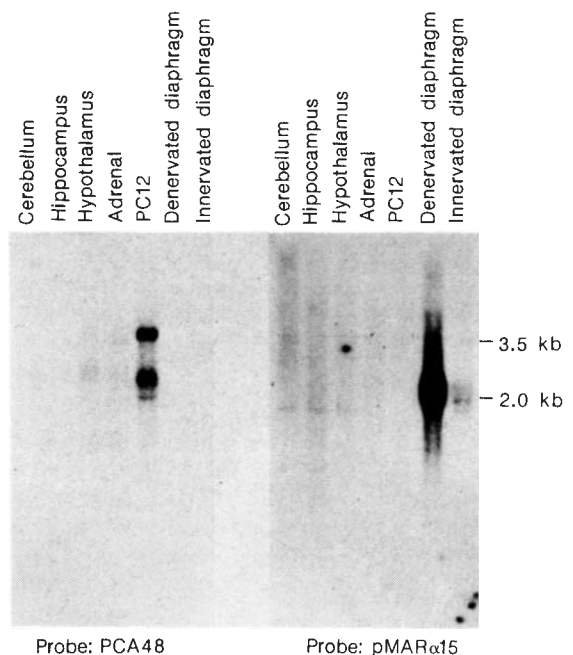


Fig. 3 Northern blot analysis of RNA isolated from Sprague-Dawley rats. Indicated regions of brain or muscle tissue were dissected, poly(A)⁺ RNA was prepared, resolved on 1.4% agarose gels and transferred to Gene Screen Plus. Filters were hybridized to probes prepared by nick-translation of λ PCA48 or pMAR α 15. Hybridization was carried out in 5 $\times$ SSPE, 50% formamide at 42 °C and the filters were washed in 0.2 $\times$ SSPE at 65 °C.

λ PCA48

The nucleotide sequence of λ PCA48 was determined over both strands. Figure 1b shows the sequence from nucleotide -225 to the 3' end together with the deduced amino-acid sequence. The sequence includes an open reading frame of 1,497 nucleotides beginning at position -75 and extending to position 1,422. This open reading frame codes for a protein that is 47% homologous to the α -subunit of the mouse muscle-type nicotinic acetylcholine receptor (see Discussion and Fig. 6). Based on an alignment of these α -subunit sequences, we identified the amino terminus of the mature protein and a leader sequence of 25 residues beginning with the methionine encoded by the ATG at nucleotide position -75. The sequence around this ATG corresponds to a favoured canonical sequence for translation initiation sites¹³ and initiation at the methionine encoded by the ATG at position -75 would produce a leader of similar length to the leaders found in other muscle acetylcholine receptor α -subunits.

Analysis of the sequence of clone λ PCA48 revealed that the 5' end of the clone is derived partly from the 5'-untranslated portion of the messenger RNA and partly from an internal sequence. The first 99 nucleotides (nucleotides -324 to -225) of the clone are the reverse and complement of the sequence beginning at nucleotide 456 and extending to nucleotide 554 in the coding portion of λ PCA48. The presence of internal sequences at the 5' end of a cDNA has been seen in other cDNA clones (unpublished observation) and probably represents a cloning artefact.

To test whether the remaining nucleotides between nucleotide -225 and the ATG at position -75 were colinear with the mRNA, the 5' *Eco*RI/*Sma*I fragment (nucleotides 1-880) was subcloned into M13mp19 and single-strand DNA prepared. This DNA was incubated with and without poly(A)⁺ RNA isolated from PC12 cells and the heteroduplexes were digested with endonuclease S_1 . The resulting fragments were denatured, fractionated on acrylamide/urea gels and electroblotted to Gene

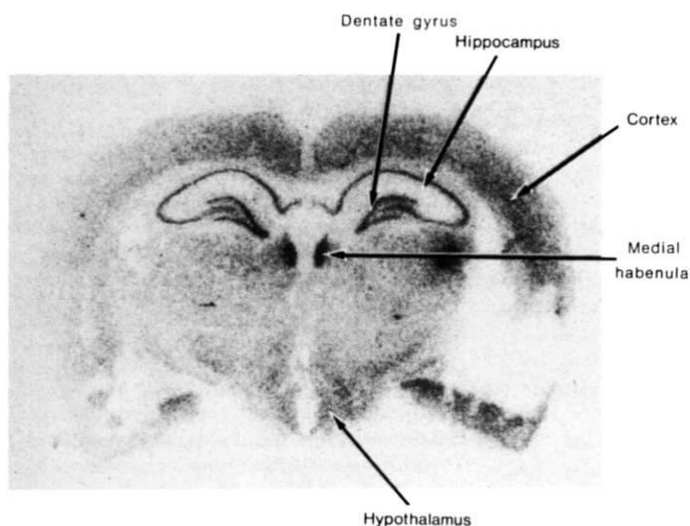


Fig. 4 Autoradiograph of *in situ* hybridization in a coronal section of a rat brain. Tissue was fixed as described by Swanson *et al.*³² and hybridization was performed using a procedure based on that described by Cox *et al.*³³. Briefly, sections (20 μ m) were mounted on polylysine-coated slides, air-dried, digested with proteinase K, acetylated with 0.25% acetic anhydride, dehydrated and hybridized with ³²P-radiolabelled single-stranded RNA probe prepared from an SP6 vector containing λ PCA48 cDNA insert. After hybridization, sections were treated with RNase A and washed at a final stringency of $0.5 \times \text{SSC}$, 42 °C. Dehydrated slides were exposed to X-ray film for 4 days.

Screen Plus. The bands in Fig. 2 were revealed by hybridization to the radiolabelled *Eco*RI/*Sma*I restriction fragment. Two DNA fragments were protected, one of which was 120 bases smaller and one which was 140 bases smaller. We conclude that there are two mRNA species homologous to λ PCA48 and that these species differ by about 20 bases at their 5' ends. Furthermore, the untranslated sequences between nucleotide -220 and the ATG at position -75 are derived from the 5'-untranslated portion of the mRNA as they are protected from endonuclease *S*₁ by the mRNA.

The open reading frame beginning at position -75 and ending at position 1,422 encodes a protein of 499 amino acids. We compared the sequence of the encoded protein with the α -subunit of the mouse muscle acetylcholine receptor (see Discussion and Fig. 6) and found considerable sequence homology. Based on this homology and the known amino terminus of the *Torpedo californica* acetylcholine receptor α -subunit¹⁴, we have placed the amino terminus of the mature protein as indicated in Fig. 1b. This results in a leader sequence of 25 amino acids and a mature protein of 474 amino acids. All the reported muscle-type acetylcholine receptor α -subunit sequences are comprised of 437 amino acids and are therefore smaller by 37 amino acids and 5,500 daltons than the 54,780-dalton protein encoded by λ PCA48. As discussed below, this additional mass is found in the proposed cytoplasmic domain located between the third membrane-spanning region and the amphipathic helix.

Although the protein encoded by λ PCA48 is homologous to the α -subunits of muscle-type nicotinic acetylcholine receptors from a variety of species, it could be a rat muscle acetylcholine receptor α -subunit, a rat neural acetylcholine receptor α -subunit or a rat bungarotoxin-binding component. We tested the first alternative by preparing poly(A)⁺ RNA from both innervated and denervated rat diaphragm muscle, size-fractionated it on agarose gels, transferred it to Gene Screen Plus and hybridized it with probes made from either λ PCA48 or pMAR α 15. Denervation of skeletal muscle has been shown to result in an increase in the abundance of acetylcholine receptors and RNA coding

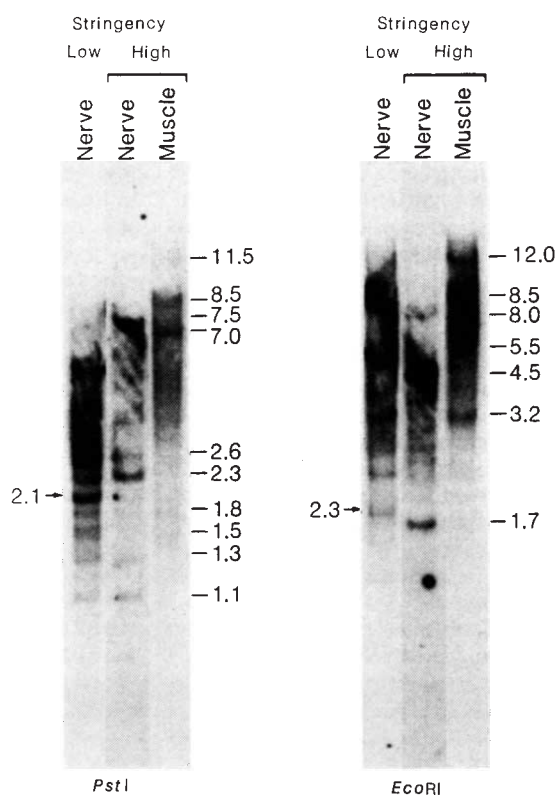


Fig. 5 Mouse genomic restriction fragments hybridizing to λ PCA48 or pMAR α 15. Genomic DNA was purified from liver of adult female C3H mice and digested with *Eco*RI or *Pst*I. The digestion products were resolved on 1.4% agarose gels, transferred to nitrocellulose and hybridized to probes prepared by nick-translation of λ PCA48 or pMAR α 15. Hybridization was carried out in $3 \times \text{SSC}$, $1 \times \text{Denhardt's}$, and $100 \mu\text{g ml}^{-1}$ carrier DNA at 65 °C. The filters were washed at 65 °C in $5 \times \text{SSC}$ (low stringency) or $0.1 \times \text{SSC}$ (high stringency). The size of the hybridizing fragments ($M_r \times 10^{-3}$) was determined from the sizes of known restriction fragments prepared from λ DNA.

for the α -subunit^{15,16}. pMAR α 15, the mouse muscle acetylcholine receptor α -subunit clone, hybridized to a 2-kb mRNA in rat muscle that increased in abundance following denervation (Fig. 3). In contrast, a probe made from λ PCA48 failed to hybridize to this mRNA species after medium-stringency washes ($2 \times \text{SSPE}$, 65 °C). In addition, poly(A)⁺ RNA from PC12 protected λ PCA48 from digestion with endonuclease *S*₁ while poly(A)⁺ RNA from rat diaphragm muscle failed to protect λ PCA48, but did protect portions of pMAR α 15 (data not shown). Therefore, the λ PCA48 corresponds to a mRNA which is different from the mRNA coding for the muscle α -subunit. We conclude that λ PCA48 does not encode the α -subunit of rat muscle nicotinic acetylcholine receptor.

If the protein encoded in λ PCA48 is a neural nicotinic acetylcholine receptor α -subunit, it should be present in neural tissue known to contain nicotinic acetylcholine receptors. Poly(A)⁺ RNA was prepared from a rat hypothalamus punch, and hippocampus, cerebellum, adrenal gland, innervated and denervated muscle, and the PC12 cell line. The RNA was fractionated on agarose gels, transferred to Gene Screen Plus and hybridized with probes prepared from either λ PCA48 or pMAR α 15. The results (Fig. 3) show that the λ PCA48 hybridizes to RNA species in all the tissues except muscle. Other experiments demonstrated that λ PCA48 hybridizes to RNA prepared from cow adrenal medulla, but not the adrenal cortex, consistent with the idea that the λ PCA48 encodes a receptor expressed in chromaffin cells (data not shown).

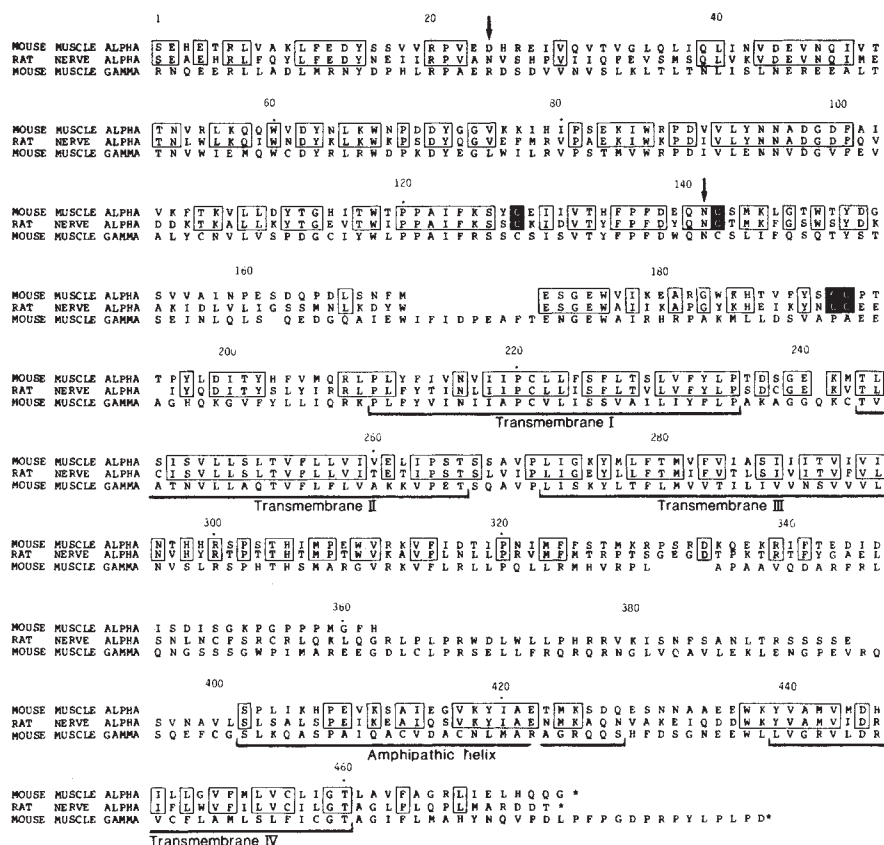


Fig. 6 Comparison of the sequences of the mouse muscle acetylcholine receptor α -subunit¹⁷ and γ -subunit¹⁸ with the rat neuronal α -subunit encoded by λ PCA48. Putative transmembrane regions and the proposed amphipathic helix are indicated. The four extracellular cysteine residues characteristic of muscle α -subunits and found in the λ PCA48 protein are shown on a black background. The arrows indicate canonical glycosylation sites. Gaps were inserted in sequences to achieve maximum alignment. The overall homology between the amino-acid sequences of the mouse muscle α -subunit and the λ PCA48 protein is 47%. At the nucleotide level the homology is 58%. Specific regions within the protein exhibit greater homology; for example, the sequences from the amino terminus to the first transmembrane region are 52% homologous (nucleotide homology, 59%), the sequences in transmembrane I are 73% (nucleotide, 75%), those in transmembrane II are 87% (74%), those in transmembrane III are 72% (67%), those in transmembrane IV are 68% (69%) and those in the amphipathic helix are 47% homologous (58%).

The regional distribution of RNA species hybridizing to λ PCA48 was also determined by *in situ* hybridization. Figure 4 shows an autoradiogram of a coronal section of a rat brain following hybridization to a probe prepared from λ PCA48. Labelling is seen in the hippocampus, the dentate gyrus and, most heavily, in the medial habenula. No such labelling is seen with probes prepared from vector sequences. This pattern of labelling is consistent with the data obtained by Northern blot analysis. In addition, the strong labelling to the medial habenula is consistent with the idea that λ PCA48 encodes the α -subunit of a neural nicotinic acetylcholine receptor, as this region of the brain binds cholinergic ligands and does not bind α -bungarotoxin¹⁷.

The above experiments demonstrate that there are RNA species homologous to the λ PCA48 clone expressed in the central nervous system. To test the possibility that RNA species homologous to the muscle-type pMAR α 15 clone also exists in the nervous system, we screened a series of tissues for homologous sequences. Northern blot analysis (Fig. 3) shows that pMAR α 15 hybridizes to RNA species extracted from the cerebellum, hippocampus, hypothalamus and adrenal gland. The size of the RNA species homologous to the pMAR α 15 probe varies in different tissues and is clearly different from the RNA species that are homologous to the λ PCA48 probe. These results suggest that genes coding for peptides homologous to the muscle-type α -subunit are expressed in the adrenal gland and the central nervous system.

Genomic sequences hybridizing to λ PCA48

The mouse muscle α -subunit and the mouse homologue of the rat nerve-type α -subunit are encoded by different genes in the mouse. Figure 5 shows a Southern blot of mouse (BALB/c) genomic DNA digested with endonucleases *Pst*I and *Eco*RI. The transferred DNA was hybridized with probes prepared using pMAR α 15 and λ PCA48 at low stringency ($3 \times \text{SSC}$, 65°C) and at high stringency ($0.1 \times \text{SSC}$, 65°C). At high stringency the

two probes hybridized to DNA fragments of different lengths, arguing that the two clones correspond to mRNAs that are the products of different genes. At low stringency the probe prepared from λ PCA48 hybridized to a number of additional restriction fragments, for example, the 2.1-kb *Pst*I fragment and the 2.3-kb *Eco*RI fragment (Fig. 6), which were not seen at high stringency in blots probed with either λ PCA48 or pMAR α 15. This suggests additional genes coding for nicotinic acetylcholine receptors exist in the mouse genome.

Discussion

Clone λ PCA48 is derived from RNA synthesized by the PC12 pheochromocytoma cell line and encodes a protein homologous to the α -subunit of muscle nicotinic acetylcholine receptor. We have shown that this clone does not encode the rat muscle nicotinic acetylcholine receptor α -subunit. The PC12 cell line expresses both a neural nicotinic acetylcholine receptor and an α -bungarotoxin binding component, either of which may be encoded by λ PCA48. The evidence that best allows us to distinguish between these alternatives comes from *in situ* hybridization data in which λ PCA48 hybridizes most consistently to brain regions known to contain cell bodies possessing dendritic processes which bind cholinergic agonists and do not bind α -bungarotoxin. These data are consistent with the idea that λ PCA48 codes for the α -subunit of a neural receptor. Furthermore, as the mRNA was derived from a cell line of probable neural crest origin, the encoded protein is likely to be an α -subunit of a ganglionic-type neural nicotinic acetylcholine receptor.

The amino-acid sequences of the mouse muscle α -subunit¹⁷, the rat neural α -subunit and the mouse muscle γ -subunit¹⁸ are aligned in Fig. 6. The amino acids are numbered with respect to their position in the neural α -subunit sequence. The muscle acetylcholine receptor α -subunits have four hydrophobic sequences which are thought to form four transmembrane regions. The protein encoded by λ PCA48 also has four hydro-

phobic sequences and in this respect is structurally similar to the muscle acetylcholine receptor α -subunits.

Finer-Moore¹⁹ and Guy²⁰ have proposed a fifth membrane-spanning region which may contribute to the ion channel in the receptor. This region is identified in Fig. 6 as the amphipathic helix. This helix partitions charged and hydrophobic amino acids such that the hydrophobic side of the helix could interact with the interior of the membrane or with hydrophobic membrane-spanning regions. The face of the helix containing the charged residues might then, through juxtaposition with homologous sequences in the other subunits, form an ion channel. The distribution of charged residues through the channel alternates with a ring of cationic residues followed by a ring of anionic residues. The protein encoded by λ PCA48 contains a sequence homologous to the amphipathic helix found in the α -subunits of the muscle nicotinic acetylcholine receptor. Although the sequence homology is not as great as is seen in the hydrophobic membrane-spanning regions, the distribution of charged and uncharged residues about the helix is similar. The conservation of this sequence between muscle and neural α -subunits supports the idea that the amphipathic helix forms a functionally important receptor domain. In fact, the single-channel conductances of rat ganglionic (44 pS)²¹ and bovine chromaffin (31 pS)²² nicotinic acetylcholine receptors are similar to the single-channel conductance of nicotinic acetylcholine receptor at the motor endplate (31 pS)²³.

All four subunits of muscle nicotinic receptor sequenced to date have a potential asparagine-linked glycosylation site at Asp 141. This site is also found in the protein encoded by λ PCA48. An additional glycosylation site occurs at Asp 24 which is not seen in the muscle α -subunit.

There is considerable evidence that both α -neurotoxins and various cholinergic agonists bind to the α -subunit of muscle acetylcholine receptor. The binding site for agonists is probably near a disulphide bond²⁴. The extracellular portion of the muscle α -subunit contains four cysteines at positions 128, 142, 192 and 193. Numa *et al.*²⁵ proposed that there is a disulphide bond between Cys 128 and Cys 142 and that the loop formed by this disulphide constitutes the acetylcholine binding site. It was subsequently proposed²⁶ that the disulphide bond is not between 128 and 142 but that there is a double disulphide bond between 128 and 192 and between 142 and 193, thus bringing into close proximity two regions of the extracellular α -subunit sequence. Kao *et al.*²⁷ have shown that an affinity labelling reagent reacts with Cys 192 and Cys 193, placing these residues in the vicinity of the acetylcholine binding site. The protein encoded by λ PCA48 also contains the four cysteines found in all the α -subunits of the muscle-type nicotinic receptor, reinforcing the idea that these residues are essential to the function of this subunit.

Although the four cysteine residues are conserved, the sequences that surround them are not. The λ PCA48-encoded protein differs from the mouse skeletal muscle receptor at 4 of the 13 residues between Cys 128 and Cys 142. Two of these substitutions, a lysine for a glutamate at position 129 and an aspartate for an isoleucine at position 130, are not conservative changes. The amino acids at these positions are conserved in all the reported muscle-type α -subunit sequences⁷. The other two substitutions are at positions which are not conserved among the α -subunits of the muscle-type receptor. If this sequence forms all or part of the acetylcholine binding site, the substitution at positions which are conserved in the muscle-type α -subunits may account for the pharmacological differences between the neural and muscle-type nicotinic acetylcholine receptors.

The sequence between Cys 142 and Cys 192 might reasonably be involved in ligand or α -neurotoxin binding, as it is contiguous with the cysteines labelled by affinity-labelling reagents. Interestingly, this sequence is one of the least conserved in the λ PCA48 protein. Specifically, the 19-residue amino-acid sequence

between residues 153 and 172 has only one residue in common with the mouse muscle α -subunit sequence. Furthermore, 8 out of the 19 residues exhibit non-conservative substitutions, replacing uncharged residues with charged residues. If this sequence is involved in ligand or toxin binding, the differences we see between the neural and muscle α -subunits could account for the fact that α -neurotoxins bind and block the muscle acetylcholine receptor but often have no effect on the neural nicotinic acetylcholine receptors. On the other hand, the region 143–191 has been proposed to form two additional membrane-spanning sequences, an extended chain membrane-spanning sequence followed by an amphipathic helix membrane-spanning region²⁸. Two observations argue against this last proposal. First, a peptide corresponding to the sequence between 173 and 204 has been synthesized and shown to bind α -bungarotoxin, suggesting that this region of the protein contributes to an α -bungarotoxin binding site²⁹. Second, the residues between 144 and 190 in the neural α -subunit are neither hydrophobic nor conserved, as are the other membrane-spanning regions in the acetylcholine receptor subunits.

The alignment of sequences seen in Fig. 6 shows an additional difference between the neural type α -subunit and the muscle-type α -subunits. In the muscle nicotinic acetylcholine receptor, the cytoplasmic domain, that is the region between the third hydrophobic membrane-spanning region and the amphipathic helix, is shorter in the α -subunit than in the β -, γ or δ -subunits. The neural-type α -subunit, however, is 37 amino acids longer than the muscle-type α -subunit and the alignment shown in Fig. 6 places these residues in the cytoplasmic domain. In this respect, the neural-type α -subunit resembles the β -, γ - and δ -subunits of muscle receptor.

Conclusions

The protein encoded by the cDNA clone λ PCA48 has a sequence that is homologous to the α -subunit of the muscle-type nicotinic acetylcholine receptor α -subunits, but differs extensively from them in two regions, one of which falls in the cytoplasmic domain of the protein while the other joins the two pairs of cysteine residues in the extracellular domain. This clone hybridizes to RNA species in neural tissue that are different from those recognized by a clone encoding the muscle-type α -subunit. The genomic restriction fragments that hybridize to the clone encoding the muscle-type α -subunit differ in size from those that hybridize to λ PCA48. In addition, at low stringency, additional restriction fragments hybridize to λ PCA48, suggesting that there are several related coding sequences in the mouse genome. A similar situation occurs in the chicken, where genomic clones have been found that code for two different α -subunits³⁰. In addition, an α -bungarotoxin binding site has been isolated from chicken brain and shown to contain a peptide with an N-terminal sequence showing both similarities to and differences from the muscle α -subunit³¹. We have made cDNA libraries from mRNA isolated from various rat brain regions and have found clones that hybridize at low stringency to probes prepared either from λ PCA48 or from pMAR α 15. This approach allows us to identify in the central nervous system a family of acetylcholine receptor molecules that are expressed in different regions of the brain. The availability of cDNA clones and the amino-acid sequence of the encoded proteins makes it possible to study these receptors in detail for the first time.

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- Patrick, J. & Stallcup, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4689-4692 (1977).
- Carbonetto, S. T., Fambrough, D. M. & Moller, K. J. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1016-1028 (1978).
- Wilson, S. P. & Kirshner, N. J. *Neurochem.* **28**, 687-695 (1977).
- Duggan, A. W., Hall, J. G. & Lee, C. Y. *Brain Res.* **107**, 166-170 (1976).
- Patrick, J. & Stallcup, W. *J. biol. Chem.* **252**, 8629 (1977).
- Greene, L. A. & Tischler, A. S. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2424-2428 (1976).
- Boulter, J. *et al. J. Neurosci.* **5**, 2545-2552 (1985).
- Gubler, U. & Hoffman, B. J. *Gene* **25**, 263-269 (1983).
- Huynh, T. V., Young, R. A. & Davis, R. W. in *gt10 and gt11 in DNA Cloning Techniques: A Practical Approach* (ed. Glover, D.) (IRL Press, Oxford, 1984).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Messing, J., Gronenborn, B., Muller-Hill, B. & Hofschneider, P. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3642-3646 (1977).
- Melton, D. A., Krieg, P. & Green, M. R. *Nucleic Acids Res.* **12**, 7035 (1984).
- Kozak, M. *Nucleic Acids Res.* **9**, 5233-5252 (1981).
- Raferty, M. A., Hunkapiller, M. W., Strader, C. D. & Hood, L. E. *Science* **208**, 1454-1457 (1980).
- Mertie, J. P., Isenberg, K. E., Russell, S. D. & Sanes, J. R. *J. Cell Biol.* **99**, 332-335 (1984).
- Goldman, D., Boulter, J., Heinemann, S. & Patrick, J. *J. Neurosci.* **5**, 2553-2558 (1985).
- Clarke, P. B. S., Schwartz, R. D., Paul, S. M., Pert, C. B. & Pert, A. J. *Neurosci.* **5**, 1307-1315 (1985).
- Boulter, J. B. *et al. J. biol. Chem.* (submitted).
- Finer-Moore, J. & Stroud, R. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 155-159 (1984).
- Guy, H. R. *Biophys. J.* **45**, 249-261 (1984).
- Rang, H. P. *J. Physiol., Lond.* **311**, 23-55 (1981).
- Fenwick, E. M., Marty, A. & Neher, E. *J. Physiol. Lond.* **331**, 577-597 (1982).
- Colquhoun, D. in *The Receptors: A Comprehensive Treatise* Vol. 1 (ed. O'Brien, R. D.) 93-142 (Plenum, New York, 1979).
- Karlin, A. & Cowburn, D. A. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3636-3640 (1973).
- Numa, S. *et al. Cold Spring Harb. Symp. quant Biol.* **48**, 57-70 (1983).
- Luyten, W., Kellaris, K., Kyte, J., Heinemann, S. & Patrick, J. *Neurosci. Abstr.* **10**, 734 (1983).
- Kao, P. N. *et al. J. biol. Chem.* **259**, 1162-1165 (1984).
- Criado, M., Hochschwender, S., Virender, S., Fox, J. L. & Lindstrom, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2004-2008 (1985).
- Wilson, P. T., Lentz, T. L. & Hawrot, E. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Mauron, A. *et al. Neurosci. Abstr.* **11**, 171 (1985).
- Conti-Tronconi, *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 5208-5212 (1985).
- Swanson, L. W., Sawchenko, P. E., Rivier, J. & Vale, W. W. *Neuroendocrinology* **36**, 165-186 (1983).
- Cox, K. H., DeLeon, D. V., Angerer, L. M. & Angerer, R. C. *Dev. Biol.* **101**, 485-502 (1984).

Expression of the *c-myb* proto-oncogene during cellular proliferation

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In several cell types, messenger RNA levels of the nuclear proto-oncogene c-myb vary as a function of cellular proliferation; a transient increase in c-myb steady-state mRNA, mediated by post-transcriptional mechanisms, occurs during cell-cycle progression. In contrast, both quiescent and proliferating immature thymocytes contain exceptionally high levels of c-myb mRNA as a consequence of increased c-myb transcription.

ALTHOUGH there is increasing evidence that cellular proliferation is controlled, at least in part, by extracellular growth factors, relatively little is known about how this control is mediated within the cell (see refs 1, 2 for reviews). The characterization of several genetic lesions resulting in arrest at specific points of the cell cycle in both yeast and several cell lines implies that there are specific regulatory (restriction) points in the growth cycle of the cell^{3,4}. A gene that exhibits a transient increase in expression during the cell cycle could, therefore, either be a regulator of a restriction point, or be responding to a cell-cycle-specific regulatory signal. Several genes involved in DNA synthesis, including dihydrofolate reductase (*dhfr*)^{5,6}, thymidine kinase (*tk*)^{7,8} and thymidylate synthetase (*ts*)⁹, undergo transient expression in the cell cycle.

Several lines of evidence suggest that proto-oncogenes are involved in the intracellular events that govern cellular proliferation¹. As many of the changes required for cell replication occur within the nucleus, one or all of the nuclear proto-oncogenes *c-myc*¹⁰, *c-fos*¹¹ and *c-myb*^{12,13} may play a regulatory role. Both *c-myc* and *c-fos* expression increase transiently following the stimulation of quiescent cells to proliferate¹⁴⁻¹⁷. However, neither of these genes shows any variation in expression within the cell cycle itself^{18,19}. It has not been determined if the third nuclear proto-oncogene *c-myb*, the cellular homologue of the transforming gene (*v-myb*) of the avian myeloblastosis virus, is involved either in the activation or in the subsequent proliferation of quiescent cells. As both the messenger RNA and protein products of *c-myb* have short half-lives^{13,20}, it is possible that changes in the expression of this gene could occur within the time-frame of these events. Here we demonstrate that *c-myb* is expressed in association with cell proliferation in various cell types. Unlike *c-myc* and *c-fos*, *c-myb* is not involved in the immediate response to serum growth factors, but does undergo a transient increase in expression that appears to correlate with cell-cycle progression in chicken embryo fibroblasts (CEF). In at least some cell types, such as MSB-1 cells (a chicken T-cell line), *c-myb* appears to

undergo a transient increase in expression in each cell cycle during exponential growth. The changes observed in *c-myb* expression during cell proliferation in both CEF and MSB-1 cells appear to be regulated primarily by post-transcriptional mechanisms. In contrast to these results, we find that the high level of expression of *c-myb* reported in immature thymic cells²¹⁻²⁵ is not cell-cycle-dependent and instead correlates with the increased transcription of *c-myb* in these cells.

c-myb expression

To examine whether the expression of *c-myb* varies with the proliferative state of the cell, we determined the amount of *c-myb* mRNA as cells in mass culture were shifted between exponential growth and growth arrest. Two types of avian cells were analysed in this way (Fig. 1): MSB-1, a transformed chicken T-cell line grown in suspension culture; and CEF. MSB-1 cells were stimulated to proliferate by diluting the cells to 2×10^5 cells per ml in fresh media. Following an initial period of exponential growth, the number of cells proliferating in culture declined until the culture entered growth arrest. The cells were then returned to exponential growth by redilution in fresh media. Total cell RNA was isolated from cells at the indicated time-points using guanidinium isothiocyanate²⁶. Northern blots were prepared from RNA samples equalized as described previously⁸ and hybridized sequentially with probes specific for the *c-myb*²⁷ and glyceraldehyde 3-phosphate dehydrogenase (*gpd*)²⁸ genes. The levels of *gpd* mRNA, a ubiquitously expressed 'housekeeping' gene, varied little. In contrast, the level of *c-myb* mRNA was highest during the period of exponential growth and decreased as the percentage of cells in the cell cycle declined in the culture over time. When the cells were restimulated to enter the cell cycle by redilution, levels of *c-myb* mRNA characteristic of exponentially growing cells were again observed. To confirm these results in a non-transformed cell type, CEF were made quiescent by density arrest. The cells were then replated at low density and cultured through exponential growth until they again reached density arrest. The

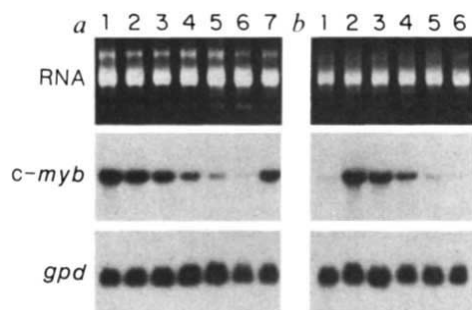


Fig. 1 *c-myb* mRNA levels in proliferating cells. **a**, MSB-1 cells; **b**, CEF cells. **a**, MSB-1 cells were cultured at 2.0×10^5 cells per ml and entered exponential growth (lane 1). They were then maintained in culture until no further increase in cell number was observed (lanes 2–6), and finally recultured at 2.0×10^5 per ml. Following reculture, the cells resumed exponential growth in 24 h (lane 7). At daily intervals as the cells went from exponential growth to saturation arrest, an aliquot was removed and total cell RNA extracted using guanidinium isothiocyanate²⁶. The samples were equalized for rRNA, and the equalization confirmed by ethidium bromide staining of equal amounts of the RNA samples separated on a non-denaturing 1% agarose gel (upper panel). These equalized RNA samples (2–10 µg) were separated on 1% agarose/formaldehyde gels and transferred to nitrocellulose. The filters were hybridized sequentially with a ³²P-labelled 1.0-kilobase (kb) *Bam*HI fragment containing a portion of the *v-myb* gene from avian myeloblastosis virus²⁷ (a gift from M. A. Baluda) and a 1.2-kb *Pst*I fragment containing a portion of the *gpd* gene²⁸. Autoradiograms were exposed for 2–48 h and equivalent exposures compared. Relative molecular masses of the mRNAs that hybridized to each of the probes agreed with previously published values^{23,28}. Quantitation was performed by densitometry. **b**, CEF cells were recultured at a density of 1×10^6 cells per 150 mm plate and maintained until confluent (lane 1). At daily intervals, as cells went from exponential growth to density arrest, an aliquot was removed and total cell RNA extracted (lanes 2–6). Samples were equalized for rRNA and Northern blots were prepared and hybridized with *v-myb* and *gpd* probes as described above. In **a** and **b**, cell viabilities as measured by trypan blue exclusion were >95%. In both cell types, >40% of the cells were in S or G₂/M phase during exponential growth and <5% had >2N DNA content during growth arrest as measured by propidium iodide staining of isolated nuclei⁸.

c-myb mRNA levels were low in quiescent cultures, increased as the cells entered exponential growth and declined as the percentage of cycling cells decreased. Again, the mRNA levels of *gpd* were relatively constant throughout.

To examine more closely the relationship between *c-myb* expression and cellular proliferation, we next analysed *c-myb* expression in serum-deprived CEF that were stimulated to proliferate by the addition of serum (Fig. 2). Under these conditions, ~80% of CEF cells will be stimulated synchronously to enter and traverse one cell cycle. After stimulation, the cells reach S phase in 10–14 h and mitosis in 20–25 h⁸. Total cell RNA was extracted after serum stimulation and Northern blots of equalized RNA were prepared and hybridized sequentially with probes specific for *c-myb*, *c-myc*⁷, histone 2B (*h2b*)⁸, *tk*⁷ and *gpd* genes. *c-myb* mRNA was first detected 2 h after serum stimulation, reached a maximum level between 4 and 8 h after stimulation, and then declined. In contrast, levels of an S-phase-expressed gene, *h2b*, did not increase until 7 h after stimulation and reached a maximum 12 h following serum addition. The *tk* mRNA is expressed with kinetics similar to *h2b*. As previously reported¹⁵, *c-myc* underwent transient induction 1 h after stimulation and then remained relatively constant over the time course of the experiment. The levels of *c-myc*, *c-myb* and *h2b* mRNA are plotted against time in Fig. 2b.

As an alternative approach to examining the relationship of *c-myb* expression to cell proliferation, levels of *c-myb* mRNA

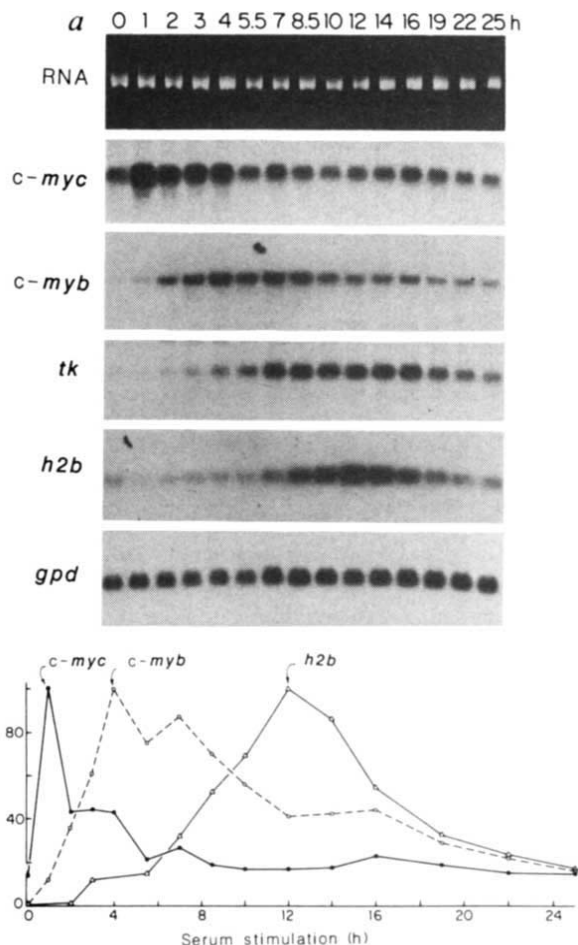


Fig. 2 **a**, The *c-myb*, *c-myc*, *tk*, *h2b* and *gpd* steady-state mRNA levels of CEF following serum deprivation and subsequent restimulation. Confluent CEF were serum deprived for 12 h (lane 1) and then stimulated to proliferate by the addition of fresh media containing 6% serum (lanes 2–15). RNA was extracted at each of the indicated time points, equalized for RNA (upper panel) and Northern blots were prepared as described in Fig. 1 legend. Filters were hybridized sequentially with nick-translated probes for *v-myb*, *c-myc*, *tk*, *h2b* and *gpd*. The *c-myc* probe was a 3.2-kb *Sst*I fragment containing the chicken *c-myc* gene⁷; the *tk* probe was a 2.9-kb *Hind*III fragment containing a portion of the *tk* gene⁷ (a gift of M. Wigler); and the *h2b* probe was a 1.1-kb *Xho*I/*Sac*II fragment containing the *h2b* gene³⁴ (a gift of P. A. Krieg). Numbers at the top of the lanes represent the time (h) after serum stimulation that RNA was extracted. **b**, Changes in *c-myb*, *c-myc* and *h2b* mRNA levels as measured by scanning densitometry and expressed as a percent of the maximal level obtained are plotted against time for the experiment described in **a**. The *gpd* levels underwent a steady increase of just under twofold through the fractions.

isolated during specific stages of the cell cycle were also examined. Exponentially growing MSB-1 cells were separated by centrifugal elutriation to enrich for populations of cells at different stages of the cell cycle (Fig. 3). Centrifugal elutriation is a counterflow centrifugation method which separates cells on the basis of volume, a parameter which correlates well with progression through the cell cycle in MSB-1 cells^{8,19}. Total RNA from each cell-cycle-dependent subpopulation was isolated and the levels of ribosomal RNA equalized to avoid the measurement of mRNA variations attributable only to increases in cell volume during growth. Northern blots of these RNAs were hybridized sequentially with the *c-myb*, *c-myc*, *h2b*, *tk* and β -actin probes. As measured by scanning densitometry, the amount of *c-myb* transcripts varied 5–10-fold through the cell cycle, with the highest levels occurring in the subpopulation most enriched in

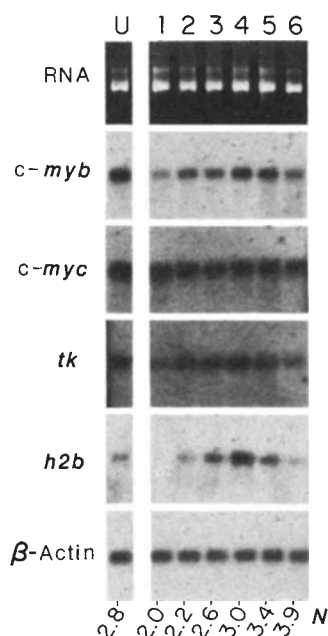


Fig. 3 The *c-myb* mRNA levels in the cell cycle. Exponentially growing MSB-1 cells were separated by counterflow centrifugation according to size⁸. Fluorescence analysis of nuclear DNA content following propidium iodide staining of cells from each fraction confirmed that an effective cell-cycle separation had occurred, cells in the first fraction had a $2n$ DNA content and cells in the last fraction had a predominantly $4n$ DNA content. RNA was extracted and equalized for rRNA levels (upper panel). A Northern blot was prepared with equal amounts of RNA from each fraction and hybridized sequentially with nick-translated probes for *v-myb*, *c-myc*, *tk*, *h2b* and the β -actin gene as described in Fig. 1 legend. Fraction numbers of the subpopulations, with the smallest cells in fraction 1 and the largest in fraction 6, are shown above the autoradiograms. The percentages of cells in each fraction were as follows: 1, 9%; 2, 21%; 3, 24%; 4, 22%; 5, 13%; 6, 11%. The β -actin probe was a 0.59-kb *Hind*III fragment containing the 3'-untranslated region of the β -actin gene⁸ (a gift from D. W. Cleveland). Lane U, unfractionated population before separation. Numbers below the autoradiograms represent average haploid DNA content (n) of the cells in each fraction.

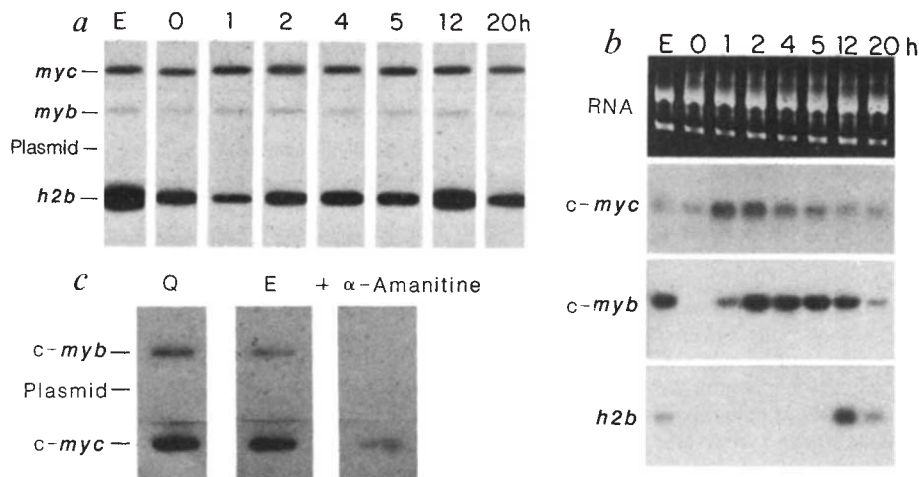
S-phase cells. In contrast, the levels of *c-myc* and β -actin mRNA varied <20% throughout the cell cycle. *h2b* appeared to be expressed only in populations of cells containing S-phase cells and *tk* underwent a variation in expression within the cell cycle similar in pattern and magnitude to that of *c-myb*. These observations represent consistent findings from several experiments involving MSB-1 cells. Using the same methods, we have also observed an increased level of *c-myb* mRNA in subpopulations enriched for S phase cells from CEF and HL-60, a human promyelocytic cell line²². In these cell types, the increase in *c-myb* mRNA is 2–3-fold rather than the 5–10-fold increase observed in MSB-1 cells. However, the level of change in *c-myb* mRNA during the cell cycle in all cell types is proportional to the cell-cycle variations observed in *tk* mRNA.

Control of *c-myb* expression

These results suggest that *c-myb* mRNA levels undergo significant variations during proliferation, either by an increase in transcription or by a stabilization of *c-myb* mRNA. To distinguish between these possibilities, the transcriptional activity of *c-myb* was examined in exponentially growing CEF, quiescent serum-deprived CEF and CEF at various times following serum stimulation of quiescent cells. From each population, nuclei were isolated and assayed by runoff transcription (Fig. 4a). No significant variation in transcriptional activity of either *c-myb* or *c-myc* was observed irrespective of the growth state of the cells. In contrast, the transcriptional activity of *h2b* was several-fold higher in exponentially growing cells than in the serum-

Fig. 4 a, The transcription rate of *c-myb*, *c-myc* and *h2b* in CEF following serum deprivation and subsequent stimulation; b, Northern blots; c, transcription rate of *c-myb* and *c-myc* in MSB-1 cells during saturation arrest (Q) and exponential growth (E).

Methods. a, CEF were serum-deprived as described in Fig. 3 legend and then stimulated to proliferate by the addition of 6% serum. At various time-points, cells in several 150-nm plates were washed with ice-cold phosphate-buffered saline and trypsinized to remove them from the plate. Nuclei were isolated and frozen at -70°C as described previously³⁵. Nuclei from different time-points were thawed simultaneously and assayed by runoff transcription as described by Groudine *et al.*³⁵ using the modifications described by Linial *et al.*³³. Equal c.p.m. of the ^{32}P -labelled transcription products were diluted in 2.25 ml high-stringency hybridization buffer¹⁶ and used to hybridize filters containing 5 μg each of indicated plasmids for 36 h at 65°C . The filters were prepared and processed after hybridization as described elsewhere¹⁶ using the following plasmids: pCM1, an Sp65 plasmid containing a 2.4-kb *Sst*I/*Eco*RI genomic fragment of *c-myc*; pHAX4, a pBR322 plasmid containing a 1.0-kb *Hae*II/*Xba*I fragment of *v-myb*²⁷; pSp65 as a plasmid control; and pH2bD1, a pSp62 plasmid including a 1.1-kb *Xho*I/*Sac*II genomic fragment including a chicken *h2b* gene³⁴. As measured by scanning densitometry, a less than twofold variation in the hybridization of either *c-myb* and *c-myc* was found between any of the time-points. b, Northern blots were prepared and hybridized as described in Fig. 3 using RNA from cells isolated at the same time as those used for the runoff transcription assay. The numbers above the autoradiograms refer to the time following serum stimulation, except that the first lane (E) was isolated from an exponentially growing control population. c, Cells in both saturation arrest and exponential growth were obtained as described in Fig. 1 legend. Nuclei were isolated and assayed by runoff transcription as described above. Addition of 2 μg ml^{-1} of α -amanitin to the runoff reaction for MSB-1 cells eliminated hybridization to the *v-myb*-containing plasmid. The small residual hybridization to the *c-myc*-containing plasmid is probably the result of nonspecific hybridization of 28S rRNA.



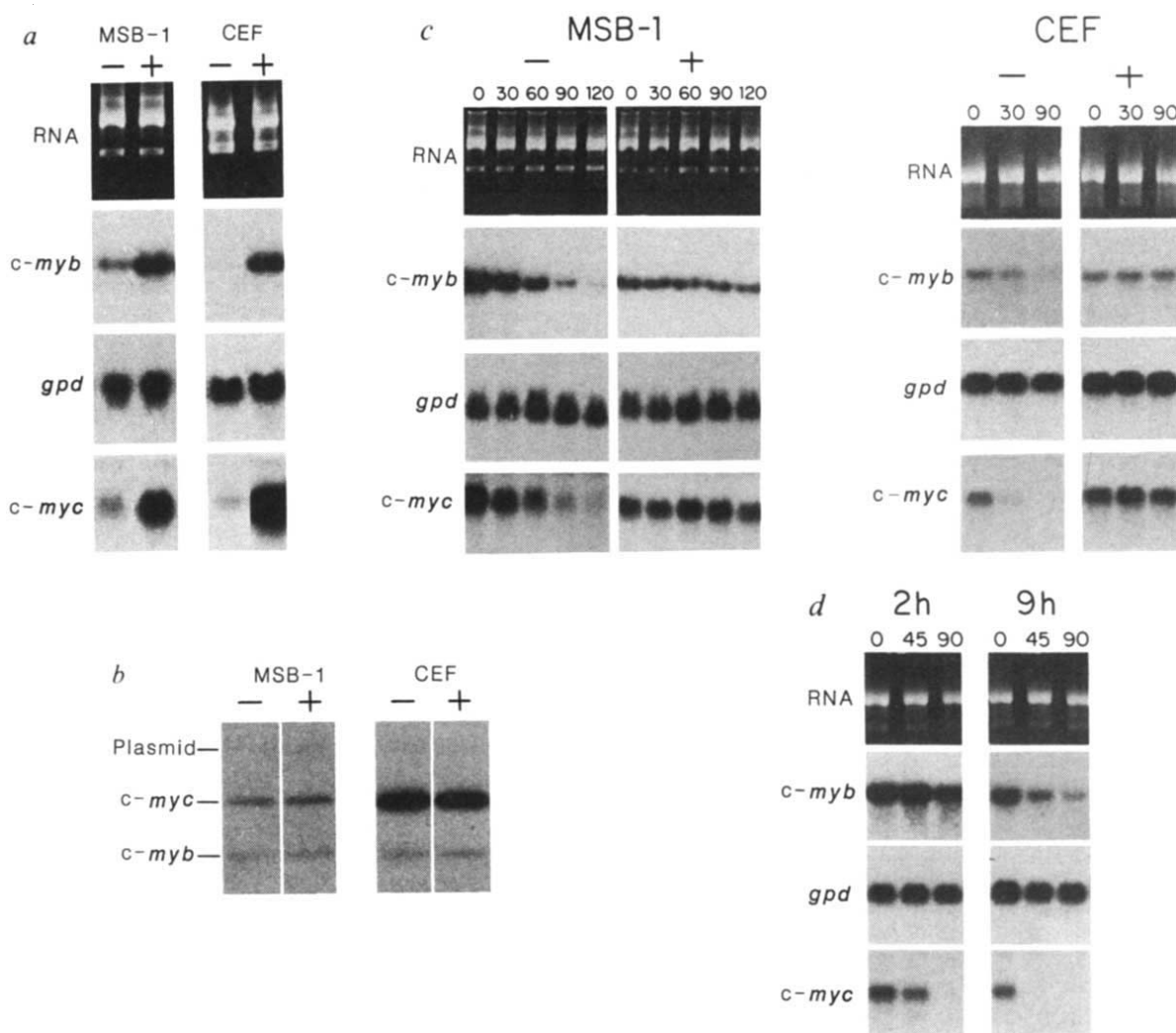


Fig. 5 *a*, The effect of cycloheximide on steady-state *c-myb* mRNA levels in CEF and MSB-1 cells. Upper panels, equalized rRNA levels; lower three panels, result of hybridizations with *c-myb*, *gpd* and *c-myc*. *b*, The effect of cycloheximide on the transcriptional rate of *c-myb* in CEF and MSB-1 cells. Upper panels, RNA equalization gels; lower three panels, hybridization with *c-myb*, *gpd* and *c-myc*. *c*, Effect of cycloheximide on the half-life of *c-myb* mRNA in CEF and MSB-1 cells. Upper panels, RNA equalization gels; lower three panels, hybridization with *c-myb*, *gpd* and *c-myc*. *d*, Half-life of *c-myb* mRNA 2 and 9 h after serum stimulation of serum-deprived CEF. Upper panels, RNA equalization gels; lower three panels, hybridization with *c-myb*, *gpd* and *c-myc*.

Methods. *a*, Quiescent CEF and MSB-1 cells were cultured for 3 h in the presence or absence of $10 \mu\text{g ml}^{-1}$ cycloheximide. RNA was isolated from the cells and Northern blots prepared and hybridized as described in Fig. 1 legend. *b*, Nuclei were prepared from cells treated for 3 h in the presence or absence of cycloheximide and runoff transcription assays performed as described in Fig. 4. The ^{32}P -labelled RNAs were hybridized to filters with $5 \mu\text{g}$ each of the *v-myb*, *c-myb* and plasmid control DNAs as described in Fig. 4 legend. *c*, The half-life of *c-myb* mRNA was measured by determining the relative amounts of steady-state mRNA following treatment of the cells with $5 \mu\text{g ml}^{-1}$ actinomycin D. To examine the effect of cycloheximide on message stability, the cells were pretreated for 30 min with $10 \mu\text{g ml}^{-1}$ cycloheximide before the addition of actinomycin D. At the indicated times following the introduction of actinomycin D, total cell RNA was extracted, the rRNA levels were equalized, and Northern blots were prepared and hybridized as described in Fig. 1 legend. Half-lives were estimated by scanning densitometry and obtaining a best linear fit for the data after log conversion of the hybridization intensity. *d*, Quiescent cells were stimulated by the addition of serum as described in Fig. 2 and $5 \mu\text{g ml}^{-1}$ of actinomycin D added at 2 and 9 h after stimulation. RNA was isolated from the cells by the guanidinium isothiocyanate method, 15 min after the addition of actinomycin D (time 0) and 45 and 90 min thereafter. The rRNAs from these samples were equalized and Northern blots prepared and hybridized as described in Fig. 1 legend.

deprived cells, increased three- to fivefold as the cells traversed S phase 12 h after serum stimulation and then decreased at 20 h as the cells entered mitosis. The total RNA isolated from the same cells confirmed that the changes in steady-state mRNA levels shown in Fig. 2 also occurred in this experiment (Fig. 4b). These results suggest that variation in *c-myb* (and *c-myc*) mRNA levels during changes in the proliferation of CEF are governed primarily by post-transcriptional mechanisms. Figure 4c shows the transcriptional activity of *c-myb* in exponentially growing and saturation-arrested MSB-1 cells. Despite the large differences in *c-myb* mRNA levels under these conditions (Fig. 1), no significant difference in transcription was observed.

Inhibition of protein synthesis by cycloheximide results in the stabilization of *c-myc* mRNA and thus causes a substantial increase of steady-state *c-myc* mRNA¹⁵. As in our experiments both *c-myc* and *c-myb* appeared to be post-transcriptionally regulated, we next determined if cycloheximide treatment had a similar effect on *c-myb* mRNA levels. When quiescent CEF or MSB-1 cells were cultured for 3 h (Fig. 5a) a 5–10-fold increase in *c-myb* mRNA was observed in cycloheximide-treated cells. As the *gpd* mRNA levels were unchanged in this experiment, cycloheximide induction of *c-myb* mRNA cannot be the result of a nonspecific increase in total mRNA. Also, the transcriptional activity of the *c-myb* and *c-myc* genes in the presence

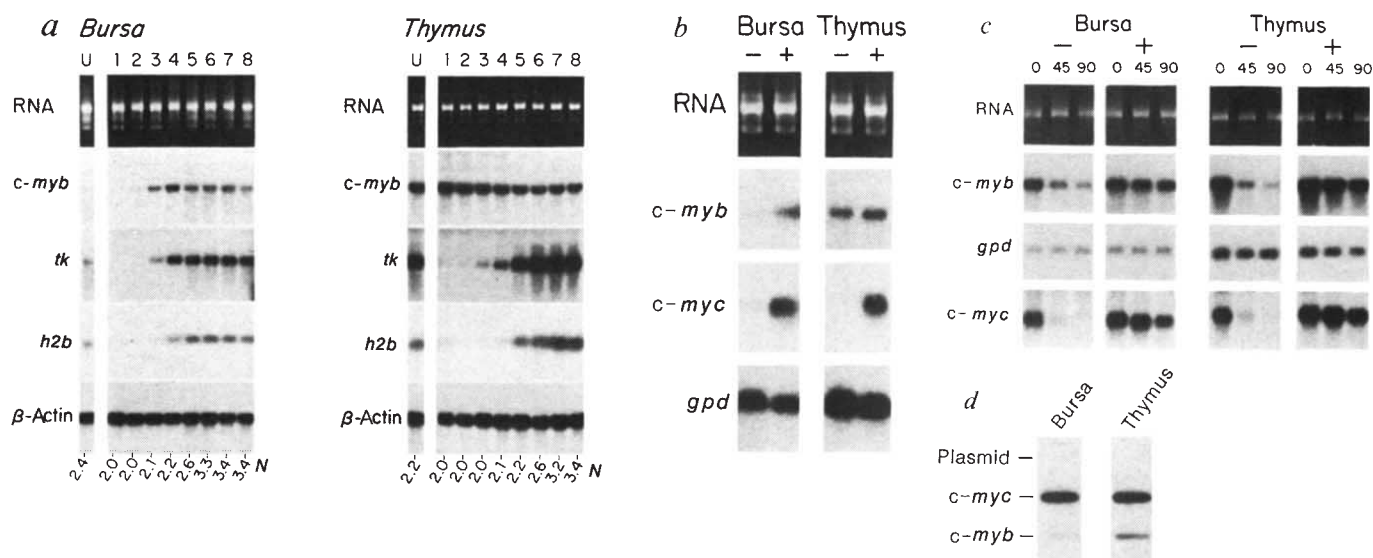


Fig. 6 *a*, The *c-myc* mRNA levels of cell-cycle-separated chicken bursal and thymus cells. Numbers at the bottom of the lanes represent the average DNA content per cell (n). Total RNA was prepared from the unfractionated thymocytes (lane U) and each of the subpopulations (lanes 1–8). The rRNA concentrations in all fractions were equalized (upper panel) and Northern blots prepared from the equalized samples as described in Fig. 1 legend. Autoradiograms of sequential hybridizations with *c-myc*, *tk*, *h2b* and β -actin are shown. *b*, Effect of cycloheximide on *c-myc* mRNA levels in bursal cells and thymocytes. *c*, Estimates of the half-life of *c-myc* in the presence or absence of $10 \mu\text{g ml}^{-1}$ cycloheximide for both bursal and thymic cells. Upper panels, RNA equalization gels; lower panels, hybridizations to *c-myc*, *c-myc* and *gpd*. *d*, Transcriptional rate of *c-myc* in bursal cells and thymocytes.

Methods. *a*, Single-cell suspensions were prepared by gently teasing apart the organs of a 6-week-old chicken and filtering the cells through nylon mesh to remove clumps. Contaminating non-lymphoid cells were removed by centrifuging the cell suspension over Ficoll-Hypaque; >95% of cells prepared from either organ in this manner stained positively with monoclonal antibodies specific for cells of the respective organ. In both organs >50% of the cells were isolated in the first three fractions, which consisted predominantly of cells with $2n$ DNA content. The average DNA content increased through the following fractions, confirming that a cell-cycle-dependent separation had occurred. *b*, Single-cell suspensions of both organs were cultured in Ham's F-10 medium containing folic acid, multivitamins and 12% serum for 3 h in the presence or absence of $10 \mu\text{g ml}^{-1}$ cycloheximide. RNA was then extracted and equalized (upper panel). Northern blots were prepared from the equalized samples and hybridized sequentially with *c-myc*, *c-myc* and *gpd* probes. *c*, Estimates were obtained using actinomycin D as described in Fig. 5c legend. At the indicated times following the addition of $5 \mu\text{g ml}^{-1}$ of actinomycin D, total cell RNA was extracted, rRNA levels were equalized, and Northern blots were prepared and hybridized as described in Fig. 1 legend. Estimates of the half-lives were obtained as described in Fig. 5 legend. *d*, Nuclei were prepared and runoff transcription assays performed as described in Fig. 4 legend. The ^{32}P -labelled RNA was hybridized to filters containing *c-myc*, *c-myc* and plasmid control DNAs.

or absence of cycloheximide was determined directly by nuclear runoff (Fig. 5b). No change in the transcriptional activity of either gene was detected following cycloheximide treatment, again suggesting that the primary control of these genes is post-transcriptional.

The simplest way to account for the observed increase in *c-myc* steady-state mRNA in the absence of a change in transcription is by stabilization of the mRNA. To investigate this possibility, the half-life of *c-myc* mRNA was determined in exponentially growing CEF and MSB-1 cells in the presence or absence of cycloheximide (Fig. 5c). Cells were treated with $5 \mu\text{g ml}^{-1}$ actinomycin D to inhibit transcription and the rate of mRNA decay was measured. Although the half-life of *c-myc* was significantly longer in CEF than in MSB-1 cells (31 min compared with 22 min), in both cell types the mRNA half-life of *c-myc* was prolonged to >200 min in the presence of cycloheximide. As reported by others²⁹, cycloheximide also significantly prolongs the half-life of *c-myc* mRNA. In contrast, cycloheximide treatment has little effect on the half-life of *gpd* mRNA. Therefore, the cycloheximide-induced increase in both *c-myc* and *c-myc* mRNA levels is caused by the stabilization of the mRNAs.

As *c-myc* levels can be significantly altered by changes in their half-life, we next determined whether or not there were differences in the half-life of *c-myc* during the accumulation and decline of *c-myc* following serum stimulation of CEF (Fig. 5d). Serum-deprived cells were stimulated by the addition of serum and the half-life of *c-myc* measured at 2 h when *c-myc* mRNA levels are rising, and at 9 h when *c-myc* mRNA levels are declining (see Fig. 2). The half-life of *c-myc* is significantly

prolonged at 2 h (246 min) compared with 9 h (28 min). The half-life of *c-myc* is also longer at 2 h (44 min) than at 9 h (18 min). This is not surprising, as the peak of *c-myc* expression following serum stimulation often does not occur until 2 h post-stimulation (see Fig. 4b; ref. 14) and also appears to be controlled by post-transcriptional mechanisms. The *gpd* mRNA half-life did not change significantly between the two time periods. At present we do not know whether the prolongation of *c-myc* and *c-myc* mRNA half-lives found 2 h after serum stimulation is specific for these genes or reflects a general prolongation of the half-lives of short-lived mRNA species. However, these results do support the conclusion that post-transcriptional regulation plays an important role in controlling *c-myc* mRNA levels during the response to proliferation.

In vivo expression of *c-myc*

To ensure that the variations in *c-myc* expression observed *in vitro* during proliferation also occur *in vivo*, we examined cell-cycle-dependent subpopulations of cells isolated from either the bursa of Fabricius or the thymus (Fig. 6). Cells from both organs were isolated as single-cell suspensions and then separated by counterflow centrifugation into size-dependent subpopulations. Total RNA was isolated from each subpopulation and used to measure steady-state mRNA levels as described for the CEF and MSB-1 experiments. In the bursa, *c-myc* mRNA levels in subpopulations containing late G_1 , S and G_2 cells were >10 times greater than in subpopulations containing the smaller G_0 and early G_1 cells. The pattern of *c-myc* expression in the bursa is similar to that of the cell-cycle-regulated genes *h2b* and *tk* (Fig. 6a).

In the thymus, *c-myb* expression did not correlate with position in the cell cycle. The level of *c-myb* mRNA was greatest in the subpopulations containing the smaller G_0 and early G_1 cells and decreased progressively in the subpopulations containing the larger cells in later stages of the cell cycle. Despite this, the patterns of expression of both *tk* and *h2b* mRNAs in the thymus were nearly identical to those found in the bursa, and the progressive increase in DNA content per cell through the fractions also confirmed that an effective cell-cycle-dependent separation of the thymus cells had occurred. β -Actin mRNA was present at similar levels in all fractions. As reported by others²¹⁻²³, we found the steady-state level of *c-myb* mRNA in the thymus to be ~10-fold greater than in any of the other cell types examined.

A qualitative difference in the regulation of *c-myb* expression between the bursa and the thymus is also shown by the effect of cycloheximide treatment on the steady-state level of *c-myb* mRNA. Bursal cells undergo a 10-fold increase in *c-myb* levels after 3 h in cycloheximide, whereas *c-myb* mRNA levels increase less than twofold in cycloheximide-treated thymocytes (Fig. 6b). In contrast to these results, the levels of *c-myc* mRNA increased more than 10-fold in both cell types. These differences in *c-myb* regulation between bursal and thymic cells do not appear to be controlled by differences in mRNA stability in the two cell types as the mRNA half-life of *c-myb* is similar in each one (30 min and 25 min, respectively), and the half-life of *c-myb* is prolonged more than sixfold in both tissues in the presence of cycloheximide (Fig. 6c). The major difference in *c-myb* regulation between bursal and thymic cells is that thymocytes have a higher absolute rate of *c-myb* transcription as assayed by nuclear runoff (Fig. 6d).

Conclusions

Our data indicate that *c-myb* is expressed during proliferation in several cell types, including CEF, MSB-1 cells and B cells from the bursa of Fabricius. In MSB-1 cells, the induction of *c-myb* expression occurs not only during the initial activation of cells to proliferate, but also in subsequent cell cycles during exponential growth. The amount of variation in *c-myb* mRNA in the cell cycle of MSB-1 cells is similar to the variations in mRNA levels reported for several other cell-cycle-regulated genes: *tk*^{7,30}, *ts*⁹ and *dhfr*⁵. For these genes, changes in mRNA levels are paralleled by variations in enzymatic activity. In addition, the expression of *c-myb* in CEF during proliferation demonstrates that *c-myb* mRNA expression is not confined to haematopoietic cells²⁴. Thus, the low levels of *c-myb* found in various tissues most likely reflects the presence of few actively proliferating cells, rather than contamination of haematopoietic cells.

The precise relationship of changes in steady-state *c-myb* mRNA levels to the cell cycle is difficult to determine. In exponentially growing MSB-1 cells, the peak of *c-myb* expression correlates well with S phase. In contrast, the increase in *c-myb* mRNA following serum stimulation of quiescent CEF occurs in late G_1 , and high levels of *c-myb* mRNA persist throughout S phase. This could reflect the difficulty in distinguishing between events in late G_1 and early S phase in exponentially growing cells separated on the basis of size. Alternatively, *c-myb* mRNA may peak during S phase, but this could be obscured in serum-deprived CEF by an earlier increase in *c-myb* mRNA levels caused by recovery from the artificial state of serum deprivation.

Regardless of the precise relationship of *c-myb* expression to position in the cell cycle, variations in *c-myb* mRNA levels during cellular activation and proliferation seem to be predominantly the result of post-transcriptional control. There is little or no change in *c-myb* transcription between exponentially growing and quiescent CEF or MSB-1 cells measured by nuclear runoff, despite the significant differences in steady-state *c-myb* mRNA levels in these conditions. Also, there is no change in

the transcription rate of *c-myb* observed in CEF following serum stimulation. In contrast, increases in *c-myb* mRNA can be induced in various cell types by cycloheximide treatment through an increase in *c-myb* mRNA stability. Changes in mRNA stability also occur following serum stimulation of quiescent CEF. The half-life of *c-myb* mRNA during the period in which *c-myb* mRNA is accumulating in the cells is significantly longer than during the period in which *c-myb* mRNA is declining (246 versus 28 min). Alteration of mRNA stability as a mechanism of gene regulation is not unique to this system. In prokaryotic cells, the level of several growth rate-dependent genes are controlled by the selective modulation of mRNA stability³¹. Two other cell-cycle-dependent eukaryotic genes, *tk* and *dhfr*, are regulated by post-transcriptional mechanisms^{5,7}. Histone genes also display a cell-cycle-dependent pattern of expression that is controlled by both changes in the rate of histone mRNA degradation and changes in the transcriptional rate during S phase³².

Previous reports concerning *c-myb* expression have described a high level of *c-myb* mRNA found in thymic lymphocytes and immature haematopoietic cells²⁰⁻²⁵. When immature myeloid cells are induced to differentiate, *c-myb* expression stops^{20,25}. As these cells stop proliferating during differentiation, decline in *c-myb* expression may simply reflect a decrease in the proliferation of these cells. Similarly, the expression of *c-myb* is high in immature thymocytes and decreases during T-cell differentiation *in vivo*²⁴. We observed levels of *c-myb* mRNA in the thymus that are at least 10-fold greater than in other lymphoid organs. Our results also suggest that the high levels of *c-myb* mRNA in the thymus are not linked to proliferation or regulated in a cell-cycle dependent manner. Thymocytes have higher levels of *c-myb* transcription than other lymphoid cells and thymic *c-myb* mRNA levels increase less than twofold following cycloheximide treatment. Thus, unlike the post-transcriptional regulation of *c-myb* expression in the cell cycle, the increased expression of *c-myb* in the thymus appears to be transcriptionally regulated. One intriguing and testable possibility is that the *c-myb* gene contains an enhancer element that responds to tissue-specific regulatory factors in the thymus. A preliminary result that supports this hypothesis is that *c-myb* transcription in the thymus decreases in response to cycloheximide treatment, suggesting that a labile protein is involved in the increased thymic transcription of *c-myb*. This could explain why cycloheximide fails to increase significantly the levels of *c-myb* mRNA in the thymus despite the observed increase in the half-life of *c-myb* mRNA following cycloheximide treatment. A precedent for this form of transcriptional regulation has been reported for the retroviral long terminal repeat enhancement of *c-myc* expression in bursal lymphoma cells³³.

We suggest that *c-myb* has both a specific function in thymic differentiation, as revealed by the high level of tissue-specific expression in the thymus, and a more general function in cellular proliferation, as characterized by the proliferation-associated expression of *c-myb* in other cells. Thus, the *c-myb* gene may have more than one role in the regulation of cellular proliferation and differentiation.

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- Heldin, C.-H. & Westermark, B. *Cell* **37**, 9-20 (1984).
- Pardee, A. B., Campisi, J. & Croy, R. G. in *Cancer Cells* Vol. 3 (eds Feramisco, J., Ozanne B. & Stiles, C.) 389-392 (Cold Spring Harbor Laboratory, New York, 1985).

3. Hartwell, L. H., Culotti, J., Pringle, J. & Reid, B. *Science* **183**, 46–51 (1974).
4. Siminovitch, L. & Thompson, L. H. *J. cell Physiol.* **95**, 361–366 (1978).
5. Leys, E. J. & Kellems, R. E. *Molec. cell Biol.* **1**, 961–971 (1981).
6. Farnham, P. J. & Schimke, R. T. *J. biol. Chem.* **260**, 7675–7680 (1985).
7. Groudine, M. & Casimir, C. *Nucleic Acids Res.* **12**, 1427–1446 (1984).
8. Thompson, C. B., Challoner, P. B., Neiman, P. E. & Groudine, M. *Nature* **314**, 363–366 (1985).
9. Storms, R. *et al. Molec. cell Biol.* **4**, 2858–2864 (1984).
10. Eisenman, R. N., Tachibana, C. Y., Abrams, H. D. & Hann, S. *Molec. cell Biol.* **5**, 114–126 (1985).
11. Curran, T., Miller, A. D., Zokas, L. & Verma, I. M. *Cell* **36**, 259–268 (1984).
12. Klempner, K.-H., Symonds, G., Evan, G. I. & Bishop, J. M. *Cell* **37**, 537–547 (1984).
13. Boyle, W. J., Lampert, M. A., Lipsick, J. S. & Baluda, M. A. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4265–4269 (1984).
14. Kelly, K., Cochran, B. H., Stiles, C. D. & Leder, P. *Cell* **35**, 603–610 (1983).
15. Greenberg, M. E. & Ziff, E. B. *Nature* **311**, 433–438 (1984).
16. Muller, R., Bravo, R., Burckhardt, J. & Curran, T. *Nature* **312**, 716–720 (1984).
17. Kruijer, W., Cooper, J. A., Hunter, T. & Verma, I. M. *Nature* **312**, 711–716 (1984).
18. Muller, R., Curran, T., Muller, D. & Guilbert, L. *Nature* **314**, 546–548 (1985).
19. Hann, S. R., Thompson, C. B. & Eisenman, R. N. *Nature* **314**, 366–369 (1985).
20. Craig, R. W. & Bloch, A. *Cancer Res.* **44**, 442–446 (1984).
21. Gonda, T. J., Sheiness, D. K. & Bishop, J. M. *Molec. cell Biol.* **2**, 617–624 (1982).
22. Westin, E. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 2194–2198 (1982).
23. Coll, J. *et al. Expl. Cell Res.* **149**, 151–162 (1983).
24. Sheiness, D. & Gardinier, M. *Molec. cell Biol.* **4**, 1206–1212 (1984).
25. Gonda, T. J. & Metcalf, D. *Nature* **310**, 249–251 (1984).
26. Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294–5299 (1979).
27. Perbal, B. & Baluda, M. A. *J. Virol.* **41**, 250–257 (1982).
28. Dugaiczky, A. *et al. Biochemistry* **22**, 1605–1613 (1983).
29. Dant, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 7046–7050 (1984).
30. Stuart, P., Ito, M., Stewart, C. & Conrad, S. E. *Molec. cell Biol.* **5**, 1490–1497 (1985).
31. Nilsson, G., Belasco, J. G., Cohen, S. N. & von Gabain, A. *Nature* **312**, 75–77 (1984).
32. Sittman, D. B., Graves, R. A. & Marzluff, W. F. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1849–1853 (1983).
33. Linial, M., Gunderson, N. & Groudine, M. *Science* **230**, 1126–1132, 1985.
34. Krieg, P. A. & Melton, D. A. *Nature* **308**, 203–206 (1984).
35. Groudine, M., Peretz, M. & Weintraub, H. *Molec. cell Biol.* **1**, 281–288 (1981).

LETTERS TO NATURE

Origin of ultra-high-energy γ -rays from Cygnus X-3 and related sources

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Ultra-high-energy (UHE) γ -ray emission from Cygnus X-3 (refs 1, 2) and the several other binary X-ray sources has been observed at γ -ray energies $\geq 10^{15}$ eV (refs 3–5). Also, collisionless shocks are expected to form in accretion flows onto neutron stars or black holes⁶. Here, therefore, we consider the diffusive shock acceleration of ions as the mechanism responsible for the γ -ray emission observed. The shock acceleration time can under reasonable assumptions be sufficiently short to allow acceleration of ions to energies near 10^{16} eV. We propose that the subsequent proton–proton (p–p) collisions and photodissociation of ^4He can produce a flux of neutrons that escapes from the acceleration site despite the high magnetic fields ($\sim 10^8$ G). These neutrons, by interacting with the binary companion^{7,8}, produce the observed UHE radiation. Radiation at such energies might be a common property of accreting binaries.

An important feature of Cyg X-3 (as well as the other sources) is that the power of the UHE emission is a sizeable fraction of the power emitted at much lower energies, that is, in X rays, indicating that the emission in the $> 10^{12}$ eV range is an integral part of the radiation-emitting mechanism. At X-ray energies, the emission almost surely originates from accretion onto a compact object. This suggests that the origin of the UHE emission may also be sought in the accretion, although alternatives such as the rotational energy of the underlying compact object⁹ or the accretion disk¹⁰ (coupled with the existing magnetic fields to produce large electrostatic potential drops) have been suggested.

Our model uses accretion as the energy source and first-order Fermi (or diffusive) shock acceleration of ions^{11–14} as the mechanism responsible for the production of the high-energy particles required to produce the observed radiation. We favour this mechanism because it is conceptually well understood, has been studied theoretically in detail, has been experimentally tested in the heliosphere^{15–17} and in galactic cosmic rays^{18,19}, it can convert a large fraction of the incoming energy flux into relativistic particles, and because shocks are ubiquitous in astrophysical environments. In particular, shocks should readily form when plasma accretes onto compact objects because the accretion velocities (of the order of the speed of light for neutron stars and black holes) are much higher than the thermal velocities of the accreting matter.

We consider that material accretes at a rate $\dot{M} = dM/dt$ onto a compact object of mass M , and that a collisionless shock (one in which the dissipation results from the elastic scattering of particles off magnetic field inhomogeneities rather than from particle–particle collisions) forms at a radius R . In normalized units we have $\dot{m} = \dot{M}/(M_{\odot} \text{ yr}^{-1})$, $M_1 = M/(10 M_{\odot})$, and the shock position, $x = R/R_s = R/(3 \times 10^6 M_1)$, where $M_{\odot}$ is the solar mass and R_s is the Schwarzschild radius. We also assume that the accretion is spherically symmetric so that the number density and ram pressure of the accreting plasma are given by

$$n \approx 10^{25} x^{-3/2} \frac{\dot{m}}{M_1^2} \text{ cm}^{-3} \quad (1)$$

$$\rho v^2 \approx 1.7 \times 10^{22} x^{-5/2} \frac{\dot{m}}{M_1^2} \text{ erg cm}^{-3} \quad (2)$$

respectively, where ρ is the density, v is the free-fall velocity, that is, $(v/c)^2 = R_s/R = x^{-1}$, and c is the speed of light.

The diffusive shock mechanism accelerates ions to a maximum energy such that either the acceleration time equals the energy loss time, or the particles escape from the accelerating region. These accelerated ions will produce neutrons: by high-energy proton–proton collisions ($p + p \rightarrow n + X$); and by the photodissociation of the UHE nuclei (mainly ^4He). We suggest that these neutrons can then, by interaction with the stellar companion of the compact object, produce the observed narrow (in-phase) UHE γ -ray pulse^{7,8}. The production of neutrons is crucial because it allows the transport of energy away from the acceleration site without invoking special magnetic field configurations (see also ref. 20). The escape of charged particles would be greatly inhibited by the presence of the strong ($\sim 10^8$ G) magnetic field inferred in our model. Furthermore, it is difficult to see how the escape of charged particles by diffusion could produce the observed narrow pulse profile. Also photons of $\sim 10^{16}$ eV produced at the acceleration site would pair produce in magnetic fields larger than $\sim 10^3$ G and would not escape either (ref. 21 and D.K., unpublished calculations). (Actually, in the $\sim 10^8$ G fields considered here, photons of energy $E \geq 10^{11}$ eV would be subject to this constraint. This argument, along with future observations in the 1 GeV–1 TeV range, might provide an estimate of the magnetic field strength.)

In high-energy p–p collisions, because of resonance formation, there is an $\sim 25\%$ probability for transforming the incoming high-energy proton into a neutron with roughly one-half the energy. (The inclusive cross-section for neutron formation in the reaction $pp \rightarrow nX$ is 50% of the total inelastic cross-section; there is also an additional 50% probability that the incoming high-energy proton will be the one to be converted into a neutron; low-energy neutrons decay *in situ* and do not contribute to the UHE flux.) Neutrons can also be produced efficiently by

the photodissociation of high-energy ${}^4\text{He}$ nuclei. This process, taking into account the cosmic ${}^4\text{He}$ abundance ($\sim$ one out of 10 nuclei is ${}^4\text{He}$), has an efficiency of $\sim 20\%$. Therefore, $\sim 50\%$ of the high-energy protons can transform into high-energy neutrons, producing a nearly isotropic neutron source. These neutrons can then, by impinging on the companion star, produce the observed radiation.

The acceleration time, τ_a , of particles to a Lorentz factor γ in diffusive shock acceleration is approximately¹⁸

$$\tau_a \approx 2.2 \times 10^{-4} \frac{A f x \gamma}{Z B} \text{ s} \quad (3)$$

where B is the magnetic field in G, f is a numerical factor (of the order 1–10) giving a measure of the particle's mean free path in gyroradii, A and Z are the ion mass and charge number, respectively, and we have used $(v/c)^2 = x^{-1}$, where v is the accretion velocity (assumed to be equal to the shock velocity).

The magnetic field B is unknown, but it can be expressed in terms of accretion quantities and the β of the accreting plasma, where $\beta = 8\pi\rho v^2/B^2$ ($\beta = 1$ for equipartition between the magnetic and particle energy densities). Assuming further that the accretion energy flux, $\frac{1}{2}\rho v^3$, is converted into radiation at the shock radius, x , we have (from equation (2))

$$\dot{m} \approx 3.5 \times 10^{-9} x L_{38} \quad (4)$$

where $L_{38} = L/10^{38}$ is the luminosity in units of $10^{38} \text{ erg s}^{-1}$. Therefore,

$$B \approx 3.9 \times 10^7 x^{-3/4} M_1^{-1} (L_{38}/\beta)^{1/2} \text{ G} \quad (5)$$

The maximum Lorentz factor achievable, γ_{max} , can then be estimated by setting the acceleration time equal to the loss time. In the conditions presently considered, the most significant ion energy loss mechanisms are: ion synchrotron; particle diffusion away from the shock; photo-pion production; and photodissociation. For shock acceleration, which demands that ions be scattered by the magnetic field, the ion synchrotron losses determine the maximum possible energy²² independent of the restrictions imposed by the other loss mechanisms.

The synchrotron loss time is

$$\begin{aligned} \tau_s &\approx 3 \frac{A^3 m_p^3 c^5}{Z^4 e^4 B^2 \gamma} \\ &\approx 6 \times 10^{18} \frac{A^3}{Z^4 B^2 \gamma} \text{ s} \end{aligned} \quad (6)$$

where m_p is the proton mass and e is the electronic charge.

Setting $\tau_a = \tau_s$ yields

$$\gamma_{\text{max}} \approx 1.7 \times 10^{11} (f x B)^{-1/2} \frac{A}{Z^{3/2}} \quad (7)$$

and substituting the value for the magnetic field in equation (5) gives

$$\gamma_{\text{max}} \approx 3 \times 10^7 M_1^{1/2} (\beta/L_{38})^{1/4} x^{-1/8} f^{-1/2} A Z^{-3/2} \quad (8)$$

Particle loss may produce a more restrictive condition if cross-field diffusion allows particles to diffuse away from the shock in a time less than the acceleration time. Using this condition, Eichler and Vestrand²³ find

$$\gamma_{\text{max}} \approx 7.1 \times 10^6 x^{-1/4} (L_{38}/\beta)^{1/2} \left(\frac{Z}{A}\right) \quad (9)$$

If synchrotron losses predominate, the maximum energy per

nucleon is a few 10^{16} eV for protons and for ${}^4\text{He}$ nuclei ($A = 4$, $Z = 2$) with $\beta = 10$ (magnetic field a factor of 3 less than equipartition), could reach $\sim 10^{17}$ eV. The secondary neutrons will be about a factor two lower in energy and can then (in identical fashion to protons as discussed by Hillas⁸) produce the observed $\sim 10^{15}$ eV γ -rays through hadronic collisions in the atmosphere of the companion star and subsequent π^0 production and decay.

Considering the simplicity of the arguments involved, the predicted maximum energy is in good agreement with the observations. This agreement becomes more significant considering the weak dependence of equation (8) on the unknown parameters of the problem. Even values of $L_{38} = 10^3$, required to account for the tentative observations of neutrino-produced muons in the IMB detector²⁴, would only change γ_{max} by a factor of ~ 5 , provided that the other parameters remain the same. The weak dependence on these parameters also implies that other X-ray binary sources would produce γ -rays of similar energies. If instead, equation (9) determines γ_{max} , this model may not generate nucleons with sufficient energy to explain the $\sim 10^{16}$ eV γ rays observed from Cyg X-3.

In both of the alternative energy loss mechanisms (photo-pion production and photodissociation, the photons of the ambient radiation field when viewed from the frame of the high-energy proton or nucleus, appear as γ -rays. These γ -rays can, in turn, either produce pions in p - γ collisions, or photodissociate the nucleus, thereby limiting the maximum attainable energy. To estimate the timescales for these processes, one needs to know the photon field of the source. The spectrum of Cyg X-3 peaks at an energy $\epsilon_m \sim 3$ keV, and is well fit by a black body of temperature $T \sim 1$ keV (ref. 25). The cross-sections for both the above processes also peak at an energy we denote as E_i ($E_i = E_\pi \approx 300$ MeV for photo-pion production and $E_i = E_d \approx 30$ MeV for ${}^4\text{He}$ photodissociation). Because of the peaked form of the target photon density, one need only calculate the timescales for a particular Lorentz factor, namely that for which $\gamma\epsilon_m \approx E_i$. Higher or lower energies would result in longer timescales. One should then compare τ_a to $\tau_{p\gamma}$ ($\gamma = E_\pi/\epsilon_m$) for photo-pion production and to τ_{dis} ($\gamma = E_d/\epsilon_m$) for photodissociation. If $\tau_a < \tau_{p\gamma}$ or τ_{dis} for the specific value of the Lorentz factor corresponding to each process, then these processes should not play a part in determining the maximum energy, which should then be given by equation (8) or (9).

For photo-pion production the energy loss rate is

$$m_p \frac{d\gamma}{dt} = \frac{L}{\pi R^2 \epsilon_m c} c (K_p \sigma)_{\text{max}} \gamma m_\pi \text{ erg s}^{-1} \quad (10)$$

where $(K_p \sigma)_{\text{max}}$ is the maximum value of the cross-section, σ , multiplied by the inelasticity, K_p , of the process²⁶, m_π is the mass of the pion, and $L/(\pi R^2 \epsilon_m c)$ is the photon number density at the peak energy, ϵ_m , of their distribution. The photo-pion production timescale is, therefore, given by

$$\tau_{p\gamma} = \frac{\gamma}{d\gamma/dt} \approx \frac{5\pi R^2 \epsilon_m}{L (K_p \sigma)_{\text{max}}} \text{ s} \quad (11)$$

Substituting the values of $\epsilon_m \sim 3$ keV, $R = xR_s$, and $(K_p \sigma)_{\text{max}} \sim 8 \times 10^{-29} \text{ cm}^2$, one obtains

$$\tau_{p\gamma} \approx 8.5 \times 10^{-5} \frac{x^2 M_1^2}{L_{38}} \text{ s} \quad (12)$$

This value agrees very well with that obtained by scaling (to a black body of temperature ~ 1 keV) the results of Protheroe²⁷, who has numerically integrated the cross-section over the photon distribution.

This timescale has to be compared with the acceleration timescale

$$\tau_a \approx 5.7 \times 10^{-12} \frac{A f x^{7/4} M_1 B^{1/2}}{Z L_{38}^{1/2}} \gamma \text{ s} \quad (13)$$

for $\gamma \sim 10^5$. It can be seen that $\tau_a \ll \tau_{p\gamma}$ for reasonable values of the parameters. At high energies, $K_p \sigma$ levels off to $\sim 5 \times 10^{-29} \text{ cm}^2$ (ref. 26) and this process will ultimately become important. Its magnitude, though, depends very sensitively on the photon number density in the acceleration region. Assuming the latter to be that of a $T \sim 1 \text{ keV}$ black body, the constraint imposed by this process is less restrictive than that of equation (8).

The same calculation can be performed for the photodissociation of ^4He by using the cross-section appropriate for this process, $\sigma_{\text{dis}} \approx 2 \times 10^{-27} \text{ cm}^2$ (ref. 28); since the peak of the cross-section is at $E_m \sim 30 \text{ MeV}$, the value of γ considered here should be $\gamma \sim 10^4$. Hence,

$$\tau_{\text{dis}} = \frac{\pi R^2 \epsilon_m}{L \sigma_{\text{dis}}} \approx 6.8 \times 10^{-7} \frac{x^2 M_1^2}{L_{38}} \text{ s} \quad (14)$$

This value is still larger than that of equation (13) for $\gamma = 10^4$, however the available margin is less and it may be possible that for certain parameters, $\tau_{\text{dis}} < \tau_a$. In this case, the efficiency of the source should be cut in half as those neutrons originating from the dissociation of ^4He would not be available at the highest energies.

Somewhat larger γ_{max} might be possible if the acceleration time is shorter than that given by equation (3). The latter result was derived assuming a single-plane parallel shock. Multiple shocks or converging magnetic fields which would reflect the particles back towards the shock might result in shorter acceleration times and thus larger maximum Lorentz factors. However, the constraint given by equation (9) must still be obeyed.

The observed photon spectrum from Cyg X-3 ($\propto E^{-2}$ photons erg^{-1}) can be obtained either by an E^{-2} input neutron spectrum extending to $\sim 10^{17} \text{ eV}$, or by an electromagnetic cascade of a high-energy ($\sim 10^{17} \text{ eV}$) monoenergetic beam as suggested by Hillas⁸. Shock acceleration can accommodate both cases. Strong classical hydrodynamic shocks have a compression ratio $r \sim 4$ and produce $E^{-(r+2)/(r-1)} \sim E^{-2}$ power law spectra¹⁸. Nonlinear shock acceleration, however, which allows for the fact that the accelerated particles may be dynamically significant, allows compression ratios larger than 4 and can produce very flat spectra at high energy^{19,29}.

Independent of the acceleration mechanism, the observation of 10^{16} -eV photons implies the existence of $\sim 10^{17}$ -eV hadrons and hence, as argued above, the existence of neutrons of approximately the same energy. It is intriguing to examine the possibility of directly observing these 10^{17} -eV neutrons. Hillas⁸ has estimated that the luminosity in the hadronic component (neutrons in our model) needed to account for the γ -ray observations is

$$L \approx 1.4 \times 10^{39} \left(\frac{\Omega}{4\pi} \right) \left(\frac{0.05}{\Delta} \right) \left(\frac{d}{10 \text{ kpc}} \right)^2 \text{ erg s}^{-1} \quad (15)$$

where Ω is the solid angle of (neutron) emission by the compact object ($\Omega = 4\pi$ in our model) and Δ is the duty cycle of the source (which could be as small as 0.02). Therefore, assuming a distance d of 10 kpc and a neutron e-folding lifetime $\tau \approx 10^3 \text{ s}$, the flux of 10^{17} -eV neutrons at the Earth, F_{17} , will be

$$F_{17} = \frac{L}{4\pi d^2 E_n} \exp \left(-\frac{m_n c d}{E_n \tau} \right) \approx 3.5 \times 10^{-17} \text{ neutrons cm}^{-2} \text{ s}^{-1} \quad (16)$$

where E_n and m_n are, respectively, the energy and mass of the neutron. This value should be compared with the cosmic ray flux above 10^{17} eV (ref. 30), that is, $F_{cr}(>10^{17} \text{ eV}) = 10^{-6} \text{ m}^{-2} \text{ h}^{-1} \text{ sr}^{-1} \approx 8.5 \times 10^{-18} \text{ cm}^{-2} \text{ s}^{-1} \text{ deg}^{-2}$. From this com-

parison it becomes apparent that an air shower array with angular resolution better than $\sim 2 \times 2$ (and with sufficient area) should be able to distinguish Cyg X-3 above the cosmic ray background at $\sim 10^{17} \text{ eV}$.

If the high-energy particle spectrum cuts off exponentially above 10^{17} eV like $E^{-2} \exp(-E/E_0)$ with $E_0 \approx 10^{17} \text{ eV}$, then at energies up to 10^{18} eV the cut-off of the particle flux is offset by the corresponding increase in the neutron lifetime to give a flux $\propto E^{-2}$. As this slope is flatter than that of the background cosmic ray flux, one should be able to improve the signal to background ratio by looking for air showers at energies near 10^{18} eV . Unfortunately, the angular resolution of these extensive air shower arrays decreases with energy and may offset this gain. The importance of such observations may warrant improving the angular resolution of high-energy arrays.

Note that even if the primary beam of particles were protons of $E \sim 10^{17} \text{ eV}$, neutrons of the same energy could still be produced when the protons interact with the atmosphere of the companion star (R. Ramaty, personal communication). The pulse profile of these neutrons should be narrow and similar to that of the 10^{16} -eV γ -rays. In contrast, the neutrons originating directly from the acceleration site near the compact object, as proposed here, should have a light curve similar to that of the X rays. The ratio of intensities in the two cases should be $\sim 1/4$ (only one out of four high-energy protons is converted into a high-energy neutron).

The neutron-initiated air showers are muon-rich and hence potentially observable with detectors sensitive to muons such as the proton decay detectors. However, the recently reported muon observations from the direction of Cyg X-3 in two of these detectors^{31,32} imply primary neutron fluxes well in excess of equation (15) and, therefore, cannot account for these observations.

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- Samorski, M. & Stamm, W. *Astrophys. J. Lett.* **268**, L17-L21 (1983).
- Lloyd-Evans, J. *et al. Nature* **305**, 784-787 (1983).
- Baltusaitis, R. M. *et al. Astrophys. J. Lett.* **293**, L69-L72 (1985).
- Protheroe, R. J., Clay, R. W. & Gerhardt, P. R. *Astrophys. J. Lett.* **280**, L47-L50 (1984).
- Protheroe, R. J. & Clay, R. W. *Nature* **315**, 205-207 (1985).
- Hawley, J. F., Smarr, L. L. & Wilson, J. R. *Astrophys. J. Suppl.* **55**, 211-246 (1984).
- Vestrand, W. T. & Eichler, D. *Astrophys. J.* **261**, 251-258 (1982).
- Hillas, A. M. *Nature* **312**, 50-51 (1984).
- Eichler, D. & Vestrand, W. T. *Nature* **307**, 613-614 (1984).
- Chanmugam, G. & Brecher, K. *Nature* **313**, 767-768 (1985).
- Krymsky, G. F. *Dokl. Akad. Nauk SSSR* **234**, 1306-1308 [*Soviet Phys. Dokl. Engl. transl.* **22**, 327-328 (1977)].
- Axford, W. I., Leer, E. & Skadron, G. *Proc. 15th int. Cosmic Ray Conf., Plovdiv* **11**, 132-137 (1977).
- Bell, A. R. *Mon. Not. R. astr. Soc.* **182**, 147-156 (1978).
- Blandford, R. D. & Ostriker, J. P. *Astrophys. J. Lett.* **221**, L29-L32 (1978).
- Lee, M. A. *Rev. Geophys. Space Phys.* **21**, 324-338 (1983).
- Ellison, D. C. *J. geophys. Res.* **90**, 29-38 (1985).
- Ellison, D. C. & Ramaty, R. *Astrophys. J.* **298**, 400-408 (1985).
- Axford, W. I. *Proc. 17th int. Cosmic Ray Conf., Paris* **12**, 155-203 (1981).
- Ellison, D. C. & Eichler, D. *Phys. Rev. Lett.* **55**, 2735-2738 (1985).
- Eichler, D. & Wiita, P. J. *Nature* **274**, 38-39 (1978).
- Stephens, S. A. & Verma, R. P. *Nature* **308**, 828-830 (1984).
- Hillas, A. M. *A. Rev. Astr. Astrophys.* **22**, 425-444 (1984).
- Eichler, D. & Vestrand, W. T. *Proc. 19th int. Cosmic Ray Conf., La Jolla* **1**, 115-118 (1985).
- Stecker, F. W., Harding, A. K. & Barnard, J. J. *Nature* **316**, 418-420 (1985).
- White, N. E. & Holt, S. S. *Astrophys. J.* **257**, 318-337 (1982).
- Stecker, F. W. *Phys. Rev. Lett.* **21**, 1016-1018 (1968).
- Protheroe, R. J. *Nature* **310**, 296-298 (1984).
- Kinsey, J. H. *Astrophys. J.* **158**, 295-302 (1969).
- Ellison, D. C. & Eichler, D. *Astrophys. J.* **286**, 691-701 (1984).
- Gaisser, T. K. & Yodh, G. B. *A. Rev. nucl. part. Sci.* **30**, 475-542 (1980).
- Marshak, M. L. *et al. Phys. Rev. Lett.* **54**, 2079-2082 (1985).
- Battistoni, G. *et al. Phys. Lett.* **155B**, 465-468 (1985).

Period derivative and orbital eccentricity of binary pulsar 1953+29

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PSR1953+29, the second fastest of more than 400 known pulsars¹, has a period of 6.133 ms and is a member of a binary system with an orbital period of 117.35 days. The system is of great interest because of what it can reveal about the evolution of pulsars and close binary stars²⁻⁴. Recently we have carried out observations to determine two of the most important parameters of the system, the pulsar period derivative $\dot{P}$ and the orbital eccentricity e . We find a small but non-zero eccentricity, $e = (330.3 \pm 0.4) \times 10^{-6}$, and a spindown rate of $\dot{P} = (1 \pm 6) \times 10^{-20} \text{ s s}^{-1}$, smaller than that measured for any other pulsar. These values are consistent with an evolutionary history involving spin-up during a mass accretion phase, but are difficult to reconcile with a recent report of periodically pulsed γ rays from this object⁵.

Observations were made with the 305-m telescopes of the Arecibo observatory on 26 different days between 16 February and 10 October 1985, during which time the pulsar completed almost exactly two orbits. At an observing frequency of 430 MHz, the Arecibo system provides a system noise temperature of 140 K and an antenna sensitivity of 14 K Jy^{-1} in each sense of circular polarization. (These figures include an allowance for 70 K of sky background noise in the direction of PSR1953+29 and for the degradation of sensitivity and increase in system noise at zenith angles near 12° .) Data acquisition is accomplished with a system built at Princeton especially for timing measurements of fast pulsars. This equipment makes use of a $2 \times 32 \times 30$ -kHz filter-bank spectrometer which provides the sums of detected, orthogonally polarized signals in each of 32 channels covering a total bandwidth of 960 kHz. Each of the 32 total-power signals is sent to a hard-wired digital signal averager which samples the intensity 128 times per period and produces an integrated pulse profile for every 1 or 2 min of observing time.

The profiles are stored on magnetic tape together with the universal time of the first sample taken in a period near the

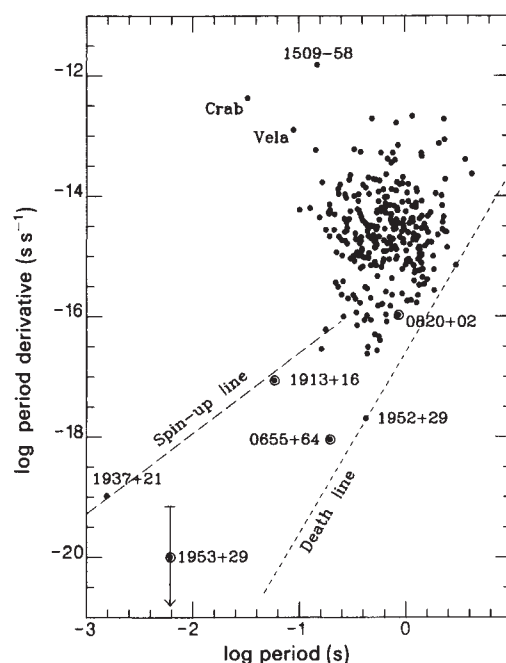


Fig. 1 Distribution of periods and period derivatives for 305 pulsars. Several pulsars of particular interest are identified, and four of the five known binary pulsars are indicated by circles around the dots. (No estimate of period derivative is yet available for the fifth binary, PSR2303+46.) The spin-up line and death line are described in the text, and original references for most of the period derivatives can be found in ref. 9. (A few measurements of $\dot{P}$ are unpublished results obtained by the Princeton University pulsar group.)

Table 1 Parameters of the PSR1953+29 system

Right ascension (1950.0)	$\alpha = 19 \text{ h } 53 \text{ min } 26.731 \pm 0.003 \text{ s}$
Declination (1950.0)	$\delta = 29^\circ 00' 43.68'' \pm 0.17''$
Pulsar period	$P = 6133166488.9 \pm 0.6 \text{ ps}$
Period derivative	$\dot{P} = (1 \pm 6) \times 10^{-20} \text{ s s}^{-1}$
Epoch of period	$t_0 = 2446113.13 \text{ JED}$
Projected semi-major axis	$a_1 \sin i = 31.41269 \pm 0.00002 \text{ light s}$
Eccentricity	$e = (330.3 \pm 0.4) \times 10^{-6}$
Orbital period	$P_b = 10138963 \pm 2 \text{ s}$
Longitude of periastron	$\omega = 29.5474 \pm 0.08 \text{ deg}$
Time of periastron	$T_0 = 2446113.0009 \pm 0.03 \text{ JED}$
Dispersion measure	$\text{DM} = 104.58 \pm 0.05 \text{ cm}^{-3} \text{ pc}$

In the table we quote uncertainties equal to three times the formal standard errors, our caution based mostly on the large covariances between P , $\dot{P}$ and δ , because our data span is shorter than a year, and between ω and T_0 , because the eccentricity is so small. DM is measurable because we included in the data set 3 days of observations made at 1,408 MHz, using a $2 \times 32 \times 250$ kHz filter bank. The error quoted for DM reflects the uncertainty in finding a reference point on a pulse profile that changes shape dramatically between 430 and 1,408 MHz⁷. The post-fit weighted r.m.s. residual for our solution is $35 \mu\text{s}$, fully consistent with the measurement uncertainties, and there is no evidence for significant non-random behavior of the residuals.

JED = Julian Ephemeris Date.

midpoint of the integration. In the first stage of data analysis, each profile is corrected to a reference frequency of 430.0 MHz, using the value of dispersion measure (DM) from Table 1, and the sets of 32 simultaneously recorded profiles are then summed. The summed profiles are cross-correlated with a standard profile, obtained by averaging many of the 2-min integrations. The observed phase delays are added to the start times of the integrations to obtain effective pulse arrival times at Arecibo. Measured pulse arrival times have uncertainties in the range 25–50 μs , depending on signal strength and zenith angle.

Subsequent analysis of the pulse times proceeded as follows. Data obtained on a given day, usually covering 20–60 min, were reduced to equivalent Solar System barycentric times with the aid of a numerical ephemeris of the Earth's motion⁶. As a first approximation we assumed the celestial coordinates of the pulsar to be those determined with the Very Large Array (VLA) telescope⁷. Because the orbital period of the system is so long, changes in orbital motion cause negligible period variations over ~ 1 h. Therefore, we obtained a best-fit pulsar period for each day's data in the reference frame of the Solar System barycentre, and used these values to carry out a least-squares solution for the binary orbital period, P_b , the time of passage through the ascending node, T_0 , the projected semimajor axis, $a_p \sin i$ (where i is the angle between the plane of the orbit and the plane of the sky) and the pulsar period in its own rest frame, P . At this stage of the analysis we could determine only an upper limit to e .

With the resulting parameters as starting values we were able to assign unambiguous pulse numbers to all the measured arrival times and thus to carry out a phase-coherent analysis of the entire data set⁸. This procedure was considerably more difficult than it has been for the other four known binary pulsars, largely because the ratio of orbit size (in light seconds) to pulsar period is much larger for PSR1953+29 than for the other pulsars. A

linearized least-squares fit to the topocentric arrival times yielded the parameter values listed in Table 1.

For comparison purposes, the values of P and $\dot{P}$ measured for PSR1953+29 are illustrated in Fig. 1, together with corresponding values for 304 other pulsars⁹. Although our measurement of $\dot{P}$ is only an upper limit, it is already the smallest known period derivative of any pulsar. Our values for the celestial coordinates are consistent with those obtained with the VLA⁷, but more accurate by factors of 20 and 4 in α (right ascension) and δ (declination), respectively. The position is consistent (at the 2σ level) with that of the candidate companion star observed by Loredó *et al.*¹⁰, although the star field is so crowded that the probability of a chance superposition within $\sim 0.3''$ is at least a few per cent.

We next summarize some astrophysical implications of the newly determined parameters of PSR1953+29. For radio pulsars, the braking torque is thought to be dominated by magnetic dipole radiation at the rotation frequency⁸, or possibly by the effects of longitudinal current flow along the open magnetic-field lines of the pulsar¹¹. Under either assumption, pulsars are expected to evolve downwards and to the right in Fig. 1, initially along lines of slope between 0 and -1 . Within a few million years, the evolutionary tracks steepen because of magnetic-field decay¹² or a tendency towards counter-alignment of the magnetic and spin axes¹¹. At the same time the radio luminosity begins to decrease¹³, and, for electro-dynamical reasons¹⁴, the pulsar will probably cease to function when it reaches a 'deathline' with a form like $\dot{P} \propto P^3$, beyond which the induced potential differences in the polar-cap region are insufficient to support electron-positron pair production. Thus, most features of the distribution of points in Fig. 1 can be understood if pulsars are born with short-to-moderate periods and large period derivatives; as they age, pulsars evolve through the general clump of points in the figure and die near the death line within $\lesssim 10^7$ yr.

The surface magnetic-field strength of a pulsar, if dipole radiation is the braking mechanism, is related to P and $\dot{P}$ by

$$B = 3.2 \times 10^{19} I_{45}^{1/2} R_6^{-3} (\dot{P} P)^{1/2}$$

where B is the field strength in gauss, I_{45} the moment of inertia in units of 10^{45} g cm² and R_6 the radius in units of 10^6 cm. With $I_{45} = R_6 = 1$, PSR1953+29 has field $< 7 \times 10^8$ G, close to the value obtained for PSR1937+21, 4×10^8 G. Such small magnetic fields imply that these objects, and others with small values of both P and $\dot{P}$, are much older than ordinary pulsars, their fields presumably having decayed from typical pulsar values of $\approx 10^{12}$ G.

Several studies have emphasized that unusually old pulsars can be accommodated in this evolutionary picture with one additional hypothesis: those born in undisrupted binary systems can be spun up by the accretion of mass and angular momentum from their companion stars (see refs 15–18 and refs therein). The angular velocity of the pulsar asymptotically approaches that of the accretion disk at its boundary with the magnetosphere. When accretion ends, the pulsar will begin to slow down according to

$$\dot{P} \approx 3 \times 10^{-20} I_{45}^{-1} (10^3 P)^{4/3} M^{5/3} \dot{m}_{17}$$

where M is the pulsar mass in solar units and $\dot{m}_{17}$ the accretion rate in units of 10^{17} g s⁻¹ (see ref. 18 for details). The spin-up line at the left of Fig. 1 illustrates this relation for $I_{45} = \dot{m}_{17} = 1$ and $M = 1.4$.

Our observations of PSR1953+29 suggest that it was 'reborn' by accreting matter from its companion. The small orbital eccentricity observed for the system implies a protracted phase of accretion accompanied by non-conservative viscous forces. Because our value of $\dot{P}$ places PSR1953+29 well below the spin-up line, it seems likely that this pulsar, like PSR0655+64, had a smaller accretion rate (or smaller mass, larger moment of inertia or some combination of these factors) than PSR1913+16. It is also possible that PSR0655+64 and PSR1953+29 are

extremely old objects that have spun down to their present P and $\dot{P}$ from initial values much closer to the spin-up line.

If the period derivative of PSR1953+29 is positive and equal to the upper limit consistent with our data, the rate of rotational energy loss is $\dot{E} = 1.3 \times 10^{34} I_{45} \text{ erg s}^{-1}$. For $I_{45} \approx 1$, and an assumed distance of 2.7 kpc, this rate is 20-fold less than the luminosity reported⁵ for the nearby γ -ray source 2CG065+0. Therefore, physical association of the pulsar with the γ -ray source seems unlikely. Although the gain of angular momentum from infalling matter could account for the very small (and possibly negative) period derivative of the pulsar an accretion powered γ -ray mechanism is inconsistent with the observed very high rotational stability. Note that our improved orbital elements can now be used to re-analyse the γ -ray data for modulation at the pulsar period without having to search over a range of possibilities.

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1. Boriakoff, V., Bucccheri, R. & Fauci, F. *Nature* **304**, 417–418 (1983).
2. Paczynski, B. *Nature* **304**, 421–422 (1983).
3. van den Heuvel, E. P. J. in *Millisecond Pulsars* (eds Reynolds, S. P. & Stinebring, D. R.) 86–103 (National Radio Astronomy Observatory, Green Bank, 1984).
4. Joss, P. C., & Rappaport, S. A. *Nature* **304**, 419–421 (1983).
5. Chadwick, P. M. *et al.* *Nature* **317**, 236–238 (1985).
6. Chandler, J. F., Babcock, R. W., Reasenberg, R. D. & Shapiro, I. I. Preprint PEP-740R (1984).
7. Boriakoff, V., Bucccheri, R., Fauci, F., Turner, K. & Davis, M. M. in *Millisecond Pulsars* (eds Reynolds, S. P. & Stinebring, D. R.) 24–29 (National Radio Astronomy Observatory, Green Bank, 1984).
8. Manchester, R. N. & Taylor, J. H. *Pulsars* (Freeman, San Francisco, 1977).
9. Manchester, R. N. & Taylor, J. H. *Astr. J.* **86**, 1953–1973 (1981).
10. Loredó, T. J., Ricker, G. R., Rappaport, S. A. & Middleditch, J. in *Millisecond Pulsars* (eds Reynolds, S. P. & Stinebring, D. R.) 48–57 (National Radio Astronomy Observatory, Green Bank, 1984).
11. Beskin, V. S., Gurevich, A. V. & Istomin, Ya. N. *Astrophys. Space Sci.* **102**, 301–326 (1984).
12. Lyne, A. G., Ritchings, R. T. & Smith, F. G. *Mon. Not. R. astr. Soc.* **171**, 579–597 (1975).
13. Lyne, A. G., Manchester, R. N. & Taylor, J. H. *Mon. Not. R. astr. Soc.* **213**, 613–639 (1985).
14. Ruderman, M. A. & Sutherland, P. G. *Astrophys. J.* **196**, 51–72 (1975).
15. Smarr, L. L. & Blandford, R. *Astrophys. J.* **207**, 574–588 (1976).
16. Blandford, R. D. & DeCampli, W. M. *IAU Symp.* No. 95 (eds Wiebeinski, R. & Sieber, W.) 371–377 (Reidel, Dordrecht, 1981).
17. Backus, P. R., Taylor, J. H. & Damashek, M. *Astrophys. J. Lett.* **255**, L63–L67 (1982).
18. Alpar, M. A., Cheng, A. F., Ruderman, M. A. & Shaham, J. *Nature* **300**, 728–730 (1982).

Feasibility of a low-mass binary source progenitor for PSR1937+214

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One suggested progenitor system for the 1.5-ms pulsar PSR1937+214 is a close binary containing, in the late stages of its evolution, a neutron star and a very-low-mass ($\lesssim 0.1 M_{\odot}$) degenerate dwarf helium star^{1,2}. The two main questions that affect the plausibility of this scenario are: (1) whether the system's evolution would in fact leave the neutron star at the observed high spin rate; and (2) whether the secondary could be disrupted, leaving a single pulsar. Here, we have analysed such a system using the predictions of Ghosh and Lamb³, and find that the equilibrium rotation period predicted for the neutron star immediately before the disruption of the secondary is so large that matter accreted from a disk after disruption would be unlikely to spin the pulsar up to the observed period. Furthermore, our estimate for the magnitude of tidal torques that transfer rotational angular momentum from an accretion disk back into the orbital motion may give values substantially higher than the previous estimate¹. It is thus unlikely that PSR1937+214 originated in a binary system in which the companion had an extremely low mass at disruption.

Among the proposed progenitors for PSR1937+214 are two involving low-mass binaries. In one version proposed by Ruderman and Shaham^{1,2,4}, a neutron star accretes matter from a low-mass companion and may be spun up to a 1.56-ms period; the companion, which becomes rich in helium during its evolution, continues to lose mass until it reaches a sufficiently low mass ($\sim 0.0053 M_\odot$)^{2,5} that it disrupts. This can only happen if the system enters a regime where angular momentum accumulates in the accretion disk around the primary. Bonsema and van den Heuvel^{5,6}, on the other hand, have proposed that the pulsar might have evolved from a neutron star binary system in which the secondary is a massive white dwarf ($\geq 0.65 M_\odot$). For a secondary mass $\geq 0.655 M_\odot$, the secondary will be disrupted regardless of whether tidal forces act to prevent angular momentum from accreting in the disk. In the first scenario, over the long time before disruption ($\sim 10^{10}$ yr), the mass transfer rate may decay by several orders of magnitude. Li *et al.*⁷ have shown that if the companion is described by the $n = \frac{3}{2}$ polytropic equation of state, and if the only angular momentum loss from the system is that due to gravitational radiation, then for a secondary of initial mass $M_2(0)$, mass fraction of hydrogen X and total system mass M (assumed conserved), we have

$$\frac{\dot{M}_2(t)}{\dot{M}_2(0)} = \left(\frac{M_2(t)}{M_2(0)} \right)^{14/3} = \frac{1}{(1+t/\tau)^{14/11}}$$

$$\tau = (1.3 \times 10^2 \text{ yr})(1+X)^{20/3} \left(\frac{M}{M_\odot} \right)^{-2/3} \left(\frac{M_2(0)}{M_\odot} \right)^{-11/3} \quad (1)$$

$$|\dot{M}_2| = (2.1 \times 10^{-3} M_\odot \text{ yr}^{-1}) \left(\frac{M}{M_\odot} \right)^{2/3} \left(\frac{M_2}{M_\odot} \right)^{14/3}$$

$$\times (1+X)^{-20/3}$$

If the primary has mass $1.4 M_\odot$, the mass transfer rate corresponding to a pure-helium $0.01 M_\odot$ secondary, twice the mass necessary for disruption, is $1.2 \times 10^{-12} M_\odot \text{ yr}^{-1}$, four orders of magnitude less than the Eddington rate. Vila's more accurate calculations based on the Zepolsky-Salpeter model⁶ show the same behaviour. The calculation of Li *et al.* assumes that no mass or angular momentum accumulates in the disk or is lost from the system over its long-timescale steady-state evolution; however, Taam and Wade⁹, who consider systems which lose mass and angular momentum by material outflow, find a similar decay unless all the angular momentum that flows into the disk is lost from the system. The equilibrium period (P_{eq}) for a spun-up neutron star is^{4,10}

$$P_{\text{eq}} = (1.57 \times 10^{-3} \text{ s}) \left(\frac{M_1}{M_\odot} \right)^{-5/7} (B_8 R_6^3)^{6/7} (\dot{M}_{-9})^{-3/7} \quad (2)$$

where B_8 is the neutron star surface magnetic field in units of 10^8 G, R_6 the neutron star radius in units of 10^6 cm, M_1 the neutron star mass and $\dot{M}_{-9}$ the mass transfer rate in units of $10^{-9} M_\odot \text{ yr}^{-1}$. The minimum neutron star radius permitted by most equations of state is ~ 7.5 km, which occurs for a neutron star mass no greater than $1.6 M_\odot$ (ref. 11). For a magnetic field of 5×10^8 G (the current estimate on the basis of measurements of P and $\dot{P}$), the equilibrium period corresponding to $1.2 \times 10^{-12} M_\odot \text{ yr}^{-1}$ must then be greater than ~ 35 ms.

The theory of Ghosh and Lamb^{3,10} treats the spin-down mechanism. The equilibrium period is achieved at a particular value of the ratio of the magnetosphere radius to the corotation radius, r_m/r_{co} . Outside r_{co} the accretion disk around the neutron star rotates more slowly than the magnetic field lines, so the magnetic coupling to the disk in this region tends to slow the neutron star down. Inside r_{co} the disk rotates more rapidly than the field lines, tending to spin the star up. A decrease in the mass transfer rate increases r_m so that more magnetic flux threads the region outside r_{co} and the star spins down. The magnetic coupling that facilitates this spin-down transfers angular momentum into the disk, from which it can be removed via tidal

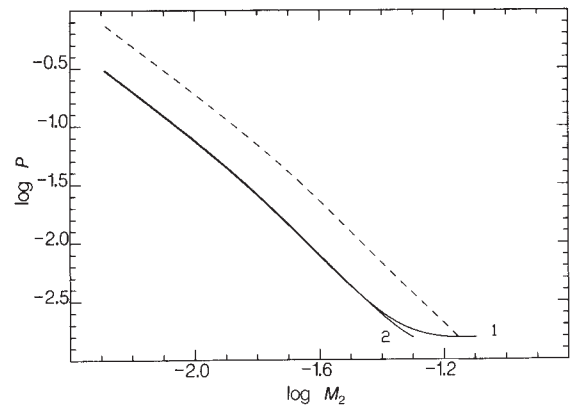


Fig. 1 Solid lines show the evolution of the period (in s) versus the secondary mass (in $M_\odot$) from integration of equation (3) using equation (1), for $B_8 = 5$, $I_{45} = 1$, $R_6 = 1$ and $M_1 \approx 1.4$. The initial value of P is 1.56 ms, and the initial values of M_2 are $0.08 M_\odot$ (curve 1) and $0.05 M_\odot$ (curve 2). For comparison, the broken line shows the result for $M_2(0) = 0.07$ if $B_8 = 14.1$, $I_{45} = 2$ and $R_6 = 1$. (This corresponds to increasing I by a factor of 2 and also increasing the constant k in the equation $BR^3 I^{-1/2} = k(P\dot{P})^{1/2}$ by a factor of 2.)

torques into the orbital motion. The equation governing the angular velocity Ω is³

$$\dot{\Omega} = (6.16 \times 10^{-3} \text{ s}^{-2}) (B_8 R_6^3)^{2/7} \left(\frac{M_1}{M_\odot} \right)^{3/7}$$

$$\times I_{45}^{-1} (\dot{M}_{-9})^{6/7} n(\omega_s) \quad (3)$$

where I_{45} is the neutron star moment of inertia in units of 10^{45} g cm^2 , $\omega_s = 0.35 \Omega/\Omega_{\text{eq}}$ is the fastness parameter [Ω_{eq} is the equilibrium angular velocity given by equation (2)] and $n(\omega_s)$ is a dimensionless torque given approximately for $0 < \omega_s < 0.9$ by³

$$n(\omega_s) = \frac{1.39}{(1-\omega_s)} \{1 - \omega_s[4.03(1-\omega_s)^{0.173} - 0.878]\} \quad (4)$$

The theory is not valid for $\omega_s \geq 0.95$. We have integrated this equation for an initial period of 1.56 ms, and find that for $B_8 = 5$, $I_{45} = 1$ and $R_6 = 1$, if the mass of the helium secondary is greater than $\sim 0.05 M_\odot$, the system evolves to a final period at disruption that is much greater than 1.56 ms (see Fig. 1); throughout this evolution the fastness parameter remains less than 0.95. The timescale for approaching the equilibrium period, given a constant mass transfer rate¹⁰,

$$\tau_\Omega = (1.2 \times 10^8 \text{ yr}) (B_8)^{-8/7} (R_6)^{-24/7} (\dot{M}_{-9})^{-3/7} I_{45} \left(\frac{M_1}{M_\odot} \right)^{2/7} \quad (5)$$

goes like $(1+t/\tau)^{6/11}$, while for the polytropic equation of state the timescale for change in the mass transfer rate is $\tau_{\text{ev}} = \dot{M}/\ddot{M} = 11/14(t+\tau)$. Thus, for large times $\tau_\Omega \ll \tau_{\text{ev}}$, and we indeed expect to observe the equilibrium period associated with the current mass transfer rate, which, as shown above, must be much larger than the initial period.

After the secondary disrupts, it will form a disk around the neutron star, some of which may be accreted and spin the neutron star up. This does not alter the above conclusions: if matter accretes from the magnetosphere boundary with the usual keplerian specific angular momentum, as several authors have established^{4,12}, $\sim 0.1 M_\odot$ would need to be transferred from the disk onto the neutron star to spin it up to 1.56 ms from a spin rate substantially below that, for the standard parameters assumed for PSR1937+214. (In Bonsema and van den Heuvel's scenario, on the other hand, the disk that results after disruption is massive enough⁶ that sufficient angular momentum can accrete from it onto the neutron star to spin the neutron star up to

1.5 ms, so that such a progenitor is feasible regardless of the behaviour of the mass transfer rate before disruption.)

We now consider the second question. The condition for the disruption of the secondary via runaway mass transfer is

$$\left| \frac{d \ln R_2}{d \ln M_2} \right| > \left| \frac{d \ln R_L}{d \ln M_2} \right| \quad (6)$$

where R_2 is the radius of the secondary and R_L the equi-volume radius of its Roche Lobe. This condition is never satisfied^{5,13,14} for $M_2 \leq 0.1 M_\odot$ unless angular momentum accumulates in the accretion disk rather than being transferred back into the orbital motion via tidal torques, in which case it is satisfied for very low masses ($\leq 0.0053 M_\odot$)^{2,5}. Ruderman and Shaham¹ have estimated that for $M_2 \ll M_1$, the tidal interaction between the disk and the secondary contributes a term of order $(M_2/M_1)^2$ to $d \ln R_L/d \ln M_2 = 1/3 - 4(M_2/3M_1)^{1/3}$ and is hence negligible. They do not present a derivation for this estimate. We have estimated the torque using equation (46) from Goldreich and Tremaine's paper on two-dimensional gas disks¹⁵. We find that the contribution to $d \ln R_L/d \ln M_2$ is of order $(M_2/M_1)^2 (M_d/\dot{M}_2 T)$ where T is the orbital period and M_d the disk mass. If, in order to produce an instability, mass must accumulate in the disk, the disk mass may come to greatly exceed the amount of matter transferred into the disk in one orbital period unless the timescale for the evolution of the instability is of the order of the orbital period. One may then not be able to discount the tidal interaction.

It is not clear how to construct a binary-origin scenario with a very-low-mass secondary in the late stages of evolution that avoids the problems of spin-down due to mass transfer rate

decay over the long time before disruption of the secondary, and of angular momentum being transferred out of the disk via tidal torques. It would seem that to prevent mass transfer rate decay, angular momentum must be lost from the system or accumulate in the disk during the long time necessary to reach the disruption mass, a rather strong condition. We suggest that without such a condition, mass transfer rate decay may render a scenario of this type unworkable. The progenitor in which the secondary is a massive white dwarf, which avoids these difficulties, may offer a better solution.

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1. Ruderman, M. A. & Shaham, J. *Nature* **304**, 425-427 (1983).
2. Ruderman, M. A. & Shaham, J. *Astrophys. J.* **289**, 244-246 (1985).
3. Ghosh, P. & Lamb, F. K. *Astrophys. J.* **234**, 296-316 (1979).
4. Alpar, M. A., Cheng, A. F., Ruderman, M. A. & Shaham, J. *Nature* **300**, 728-730 (1982).
5. Bonsema, P. F. J. & van den Heuvel, E. P. J. *Astr. Astrophys.* **146**, L3-L5 (1985).
6. van den Heuvel, E. P. J. & Bonsema, P. T. J. *Astr. Astrophys.* **139**, L16-L18 (1984).
7. Li, F. K., Joss, P. C., McClintock, J. E., Rappaport, S. & Wright, E. L. *Astrophys. J.* **240**, 628-635 (1980).
8. Vila, S. C. *Astrophys. J.* **168**, 217-223 (1971).
9. Taam, R. E. & Wade, R. A. *Astrophys. J.* **293**, 504-507 (1985).
10. Henrichs, H. F. in *Accretion-Driven Stellar X-ray Sources* (eds Lewin, W. H. G. & van den Heuvel, E. P. J.) 393 (Cambridge University Press, 1983).
11. Shapiro, S. L. & Teukolsky, S. A. *Black Holes, White Dwarfs and Neutron Stars* (Wiley, New York, 1983).
12. Henrichs, H. F. & van den Heuvel, E. P. J. *Nature* **303**, 213-215 (1983).
13. Hut, P. & Paczynski, B. *Astrophys. J.* **284**, 675-684 (1984).
14. van den Heuvel, E. P. J. in *Proc. Green Bank Workshop on Millisecond Pulsars* (eds Reynolds, S. & Stinebring, D.) 86-103 (National Radio Astronomy Observatory, Green Bank, West Virginia, 1984).
15. Goldreich, P. & Tremaine, S. *Astrophys. J.* **233**, 857-871 (1979).

Long-term changes in the semimajor axes of the outer planets

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One of the oldest problems of celestial mechanics is that of the long-term behaviour of the semimajor axes a of the planetary orbits. Analytical theories^{1,2} predict periodic variations in a , some of which may have very long periods, but these terms have never been computed. We have now performed a 9.3-Myr numerical integration of the orbits of the outer planets, using a pure newtonian point mass model. An accurate integrator and an effective low-pass filtering of the output allow us to detect high-order variations in the energies, and hence also in a , with periods ranging from tens of thousand to millions of years. The most interesting feature is an energy exchange between Uranus and Neptune with a period of 1,119,000 years, the same as the period of the libration between the perihelia of Jupiter and Uranus^{3,4}. The mechanism involves Jupiter and also Saturn; moreover, their energy shows puzzling longer-term trends. The energy of Pluto changes mostly with periods close to that of the 3:2 libration in mean motion with Neptune. Its spectrum in this region shows a very complicated structure; however, we have found no indication of chaotic behaviour.

Two centuries ago, Lagrange showed that to a first order in the masses μ of the perturbing planets (in units of the mass of the Sun; $M_\odot = 10^{-3}$ at most, in the case of Jupiter), the semimajor axes are subject only to short periodic changes whose frequencies are linear combinations of the mean motions and which show neither secular nor long-period changes. Message^{1,2},

following Poincaré⁵, showed that to any order in μ the semimajor axes have periodic changes only, some with the very long periods of the quantities e , i , $\bar{\omega}$, Ω (eccentricity, inclination, longitude of the pericentre and longitude of the node respectively) which describe the shape and orientation of the orbit. On the other hand, according to Poisson, the time derivative of a has neither secular nor long-period terms to the order μ^2 . We conclude that the largest long-period variations in a must be of the order of μ^3 divided by a 'small divisor' which is the frequency of the quantities e , i , $\bar{\omega}$, Ω . These frequencies are zero in the degenerate problem in which the pericentres and the nodes are not perturbed by the neighbouring planets, and are of the order of μ in the real problem. Therefore, we searched for long-period variations in a of the order of μ^2 , that is, of relative amplitude 10^{-6} or smaller.

Within the Longstop project, we performed a numerical integration of the five outer planets for 9.3 Myr on the Cray 1S at the University of London Computer Centre. We used a new numerical integrator (Encke's method with non-restarting rectification; A.M., N.M.N. and M.C., manuscript in preparation); the relative variation of the total energy at the end of the integration is only $\approx 3 \times 10^{-10}$. Because the energies of the planets have short-period variations of the order of μ , they must be filtered out so that long-period variations can be detected. We designed an *ad hoc* low-pass filter with a pass band for periods $> 10,000$ yr, a dark band between 11 and 5,000 yr, and a gap (where the performance of the filter is poor) between 5,000 and 10,000 yr, where it is known that the spectrum of the Solar System is devoid of lines. The attenuation factor in the dark band is 10^{-4} , smaller than μ , so we can be sure that the short-period terms become smaller than the long-period ones. The filter was applied concurrently with the integration, and the initial conditions were fitted to the Nautical Almanac ephemerides (A. T. Sinclair, personal communication). The effect of the inner planets was taken into account only by adding their mass to that of the Sun. Details will be presented elsewhere (A.M., A.M.N. and M.C., manuscript in preparation).

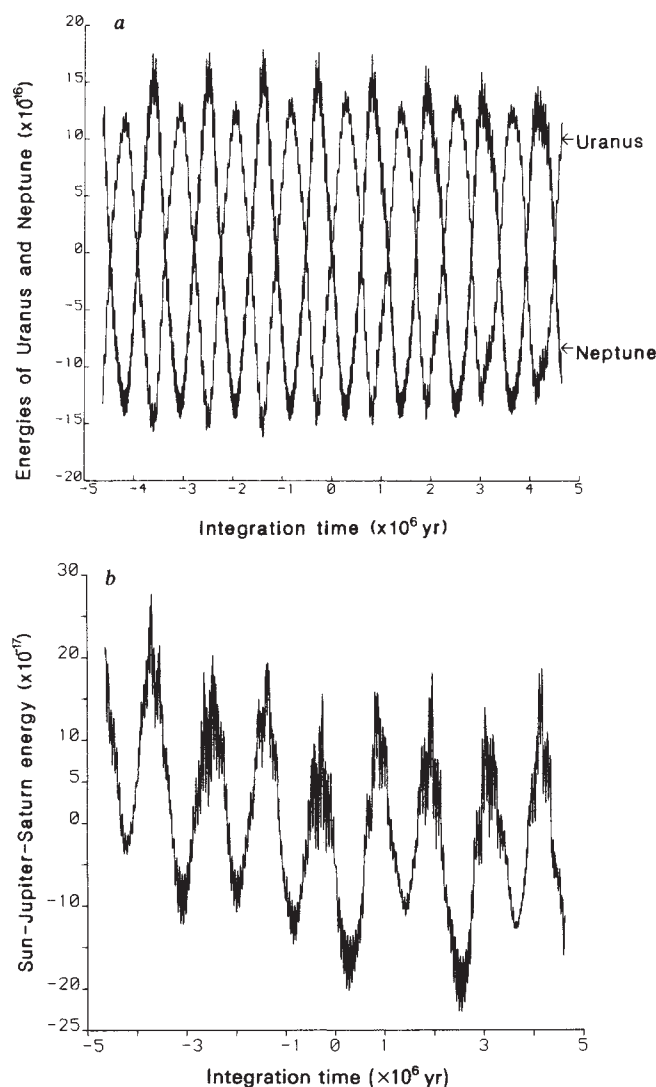


Fig. 1 *a*, Energies of Uranus and Neptune, plotted as deviations from their averages (respectively -3.36×10^{-10} and -2.52×10^{-10} in the units used here, that is, solar masses $\times$ astronomical units² $\times$ days⁻²). The main features in both curves are oscillations with a period of 1.119 Myr, almost exactly in anti-phase. The higher-frequency ripple in Neptune's curve is caused by the resonant interaction with Pluto. *b*, Energy of Jupiter and Saturn together (that is including their mutual perturbations) plotted as deviation from the average value of -3.16×10^{-8} . The Sun-Jupiter-Saturn subsystem exchanges energy with the outermost planets over the same period of 1.119 Myr with which Uranus and Neptune exchange energy with each other. There is also a clear indication of a longer-period term.

The most interesting result is shown in Fig. 1, where the energies of Uranus and Neptune are plotted as functions of time (4.6 Myr forwards and 4.6 Myr backwards from the present). There is an exchange of energy (relative energy exchanged $\approx 5 \times 10^{-6}$) with a period of 1.119 Myr; this is also the period over which Jupiter and Uranus exchange angular momentum and it is known to be essential for maintaining the stability of the system^{3,4}. The energy of Jupiter shows a dominant variation with period 54,000 yr (the mutual period of the perihelia of Jupiter and Saturn) modulated over 1.119 Myr. The same is true for Saturn. However, the energy of the Sun-Jupiter-Saturn three-body subsystem⁴ (including their mutual perturbations) shows a dominant 1.119-Myr oscillation, in phase with that of Neptune, with an indication of a longer-period term (Fig. 2a). Figure 2b plots the total energy of the system, showing the round-off error, and we can see that the long-term trend

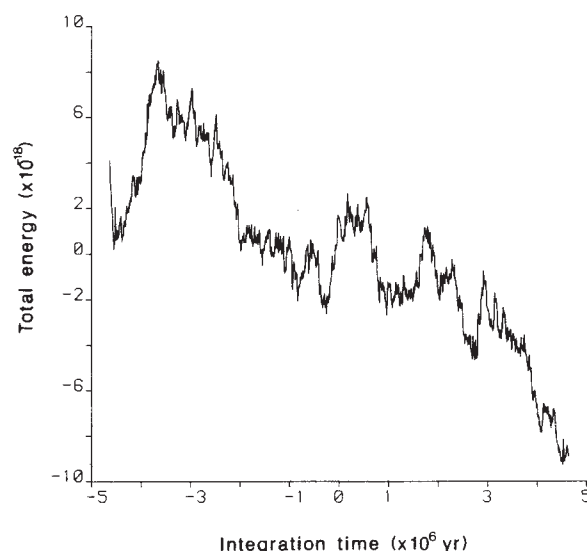


Fig. 2 The total energy as a function of time computed from the output of our numerical integration (deviations from the average value of -3.22×10^{-8}). The rounding-off error is mostly responsible for the slow pseudo-random drift of this function, which would be constant along the exact solution. The ostensibly linear trend (of $\sim 10^{-17}$ over 9.3 Myr) is, however, smaller than all the features shown in the other figures.

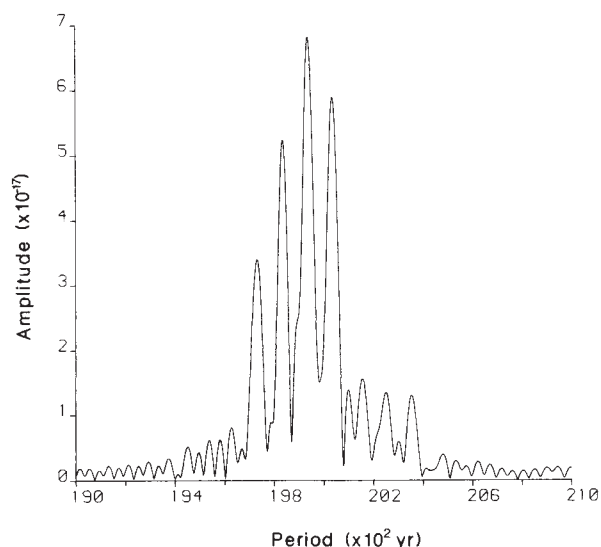


Fig. 3 An enlarged view from the spectrum of the energy of Pluto. The main lines are separated in frequency by multiples of the frequency of libration of Pluto's argument of perihelion⁸.

apparent in Fig. 2a is not caused by the numerical error, because the latter is roughly 10 times smaller.

Figure 3 shows the spectrum of Pluto's energy around the 19,900-yr period of the 3:2 resonance in the mean motion with Neptune. The finding that the spectral line corresponding to the critical argument of this resonance is split into several evenly spaced lines suggests the possible existence of a 'modulational layer'⁶ where resonances overlap. If this is the case, a slow diffusive change in the orbital elements could occur and a chaotic behaviour could appear in the long term. However, the integration time is too short to distinguish between a continuous and a discrete spectrum. Pluto's dynamical behaviour seems to be regular in 9.3 Myr and the usual test of the divergence of nearby orbits⁷ also shows no indication of instability.

Our analysis, using the same methods, of a previous 5-Myr integration⁸ gives consistent results. At present there is no

explicit analytical theory on the long-term behaviour of the semimajor axes with which these results can be compared. A comparison is possible for the quantities e , i , $\bar{\omega}$ and Ω and shows quite good agreement for first-order terms, although serious differences arise for the higher-order ones.

A time of 9.3 Myr is still very short compared with the age of the Solar System, and longer numerical integrations are needed; we and others⁹ are working in this direction. For longer integrations to be significant, the round-off error must be carefully controlled to ensure that it will not mask important dynamical features; furthermore, a better physical model must be used to represent the real problem. We are about to start a 100-Myr integration of the outer planets using the same numerical method but improving the physical model. The secular perturbations caused by the inner planets on the perihelia and the nodes of the outer planets are significant over such timescales (for example, the effect of the Earth on the perihelion of Jupiter in 50 Myr is $\sim 88^\circ$). General relativistic corrections are small but not negligible¹⁰. Moreover, the relative mass loss of the Sun through electromagnetic emission only produces a relative semimajor axis increase of $6.6 \times 10^{-14} \text{ yr}^{-1}$. In our integration this effect is already three orders of magnitude larger than the round-off error. However, one might argue that the mass loss

of the Sun will simply rescale the orbits and the periods of the planets and therefore is not likely to have any significant effect on the dynamical structure of the whole system, whereas the numerical errors act in different ways on the different planets and therefore in the long run will affect the dynamical structure. We conclude that the control of the numerical errors, especially the round-off, is at present our most serious problem in elucidating the stability of the Solar System.

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1. Message, P. J. in *Long-Time Predictions in Dynamics* (eds Szebehely, V. & Tapley, B.) 279–293 (Reidel, Dordrecht, 1976).
2. Message, P. J. *Celestial Mech.* **26**, 25–39 (1982).
3. Milani, A. & Nobili, A. M. *Nature* **310**, 753–755 (1984).
4. Milani, A. & Nobili, A. M. *Celestial Mech.* **35**, 269–287 (1985).
5. Poincaré, H. *Methodes Nouvelle de la Mechanique Celeste* Vol. 2 (Gauthier-Villars, Paris, 1893).
6. Vivaldi, F. *Rev. mod. Phys.* **96**, 737–754 (1984).
7. Benettin, G., Galgani, L. & Strelcyn, J. M. *Physical Review A* **14**, 2338–2345 (1976).
8. Kinoshita, H. & Nakai, H. *Celestial Mech.* **34**, 203–217 (1984).
9. Applegate, J. F. *et al. IEEE Trans. Computers* **c34**, 822–831 (1985).
10. Nobili, A. M. & Roxburgh, I. W. *Proc. IAU Symp.* **114** (in the press).

D to H ratio and the origin and evolution of Titan's atmosphere

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A value of 1.7×10^{-3} has been reported¹ for the ratio of CH_3D to CH_4 in the stratosphere of the saturnian moon Titan. A lower value of 6×10^{-4} for this ratio in the deeper part of Titan's atmosphere was reported by de Bergh *et al.*². For comparison^{3–11} we note that the CH_3D to CH_4 ratio on Saturn and Jupiter is 7.7×10^{-5} and 6.7×10^{-5} , respectively (see Table 1). We estimate the uncertainties in all these observations and data reduction to be about a factor of 2. Despite these uncertainties it appears that Titan's atmosphere is enriched in deuterium by a factor of ≥ 3 relative to Jupiter and Saturn. Potential causative factors examined here for this enrichment are condensation to form tropospheric methane clouds, fractionation occurring over a hypothetical CH_4 – C_2H_6 ocean and between the ocean and the clathrate crust beneath, fractionation which occurred during the formation of Titan and fractionation occurring as a result of the evolution of Titan's atmosphere. We conclude that the greater part of the observed fractionation is probably derived from the formation of Titan and the subsequent evolution of Titan's atmosphere driven by photochemistry.

We first consider possible fractionation that occurs during formation of tropospheric methane clouds. Data in ref. 12 indicate the CH_4 – CH_3D liquid solution to be ideal in the sense that the ratio of components in the gas phase is equal to that in the liquid, multiplied by the saturation vapour pressure ratio. As the saturation vapour pressures of CH_4 and CH_3D are within 2% of the temperature range of Titan's tropopause, we conclude

that no fractionation effects greater than a few per cent can occur with cloud formation.

Of greater concern are the relative solubilities of CH_4 and CH_3D in the postulated ethane–methane ocean¹³. In the limit that the CH_3D – CH_4 solution is considered as a single component of the ethane–methane system, the $\text{CH}_3\text{D}/\text{CH}_4$ ratio in the ocean would be within several per cent of that in the corresponding vapour, based on the preceding argument. Alternatively, the deuterated methane could be regarded as a distinct dilute component in the ocean, and its solubility computed from data on binary mixtures. Using data on CH_4 – C_2H_6 mixtures^{14–16} extrapolated to extreme dilution, and assuming the solubility of CH_3D in C_2H_6 is the same for a fixed composition as that of CH_4 in C_2H_6 , we find $(\text{CH}_3\text{D}/\text{CH}_4)_{\text{atmosphere}}/(\text{CH}_3\text{D}/\text{CH}_4)_{\text{ocean}} \approx 1.3$. We cannot rule out either limiting estimate in the absence of experimental data, and hence conclude that the enrichment factor is in the range 1.0–1.3.

The ocean is likely to be underlain by a water-ice crust¹⁷; hence, clathrate formation should occur at the interface. The relative extent of incorporation of molecules of similar shape in the clathrate structure tends to follow their vapour pressure¹⁸; since these are nearly identical for CH_3D and CH_4 no partitioning is expected. In any event, the mass of the clathrate layer in contact with the ocean is estimated to be small compared with that of the ocean¹⁷.

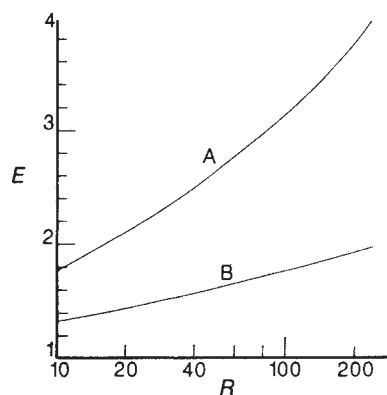
Little is known about the D/H ratio in the primordial gaseous nebula from which satellites formed, because this ratio is sensitive to physical and chemical processes involved in satellite formation which are not well quantified. In a gas containing cosmic abundances of H, O, C and N molecular species, the substitution of a deuterium for a hydrogen in heavier molecules leads to an increase in the binding energy, which becomes increasingly important as the thermal energy per molecule decreases. According to Hubbard and MacFarlane¹⁹ the D/H enhancement for methane in equilibrium with H_2 is about 2 at 500 K and can be as large as 7 at 200 K. Opposing such enhancements are the rather long equilibration times required in the gas phase. Estimates by Beer and Taylor²⁰ suggest that for $\tau \leq 500$ K the equilibration time would exceed 10^8 yr, which is considerably longer than the timescale for satellite formation^{21,22}. Catalysis on the surface of metal rich grains may lower the equilibration times^{23–27}. Recent calculations of D. H. Grinspoon and J. S. Lewis (personal communication) suggest that surface catalysis

Table 1 Observations of the relative abundances of deuterium and hydrogen in planetary atmospheres

Planetary body	Relevant physical quantity	Notes
Titan	$\text{CH}_3\text{D}/\text{CH}_4 = 1.7 \times 10^{-3}$	Ref. 1, based on ν_6 band of CH_3D at $8.6 \mu\text{m}$; Voyager IRIS data ³
	$\text{CH}_3\text{D}/\text{CH}_4 = 6 \times 10^{-4}$	Ref. 2, personal communication based on $6,425 \text{ cm}^{-1}$ band of CH_3D ; ground-based data; result probably more certain than that using ν_6 emission feature
Saturn	$\text{CH}_3\text{D}/\text{CH}_4 = 8.7 \times 10^{-5}$	Ref. 5, based on ν_6 band of CH_3D ; Voyager IRIS data
Jupiter	$\text{CH}_3\text{D}/\text{CH}_4 = 6.7 \times 10^{-5}$	Refs 4 and 5, based on ν_6 band of CH_3D ; Voyager IRIS data; ref. 11
Earth	$4 \times (\text{D}/\text{H}) = 6.2 \times 10^{-4}$	Ref. 8, based on standard mean ocean water (SMOW); for juvenile water see ref. 9
Venus	$4 \times (\text{D}/\text{H}) = 6.4 \times 10^{-2}$	Refs 6 and 7, based on D^+ and HDO measurements on Pioneer Venus
Mars	$4 \times (\text{D}/\text{H}) = 6.2 \times 10^{-4}$	Ref. 10, based on $\delta\text{D} \sim -80$ to 52% in Shergotty meteorite; connection with the martian atmosphere controversial

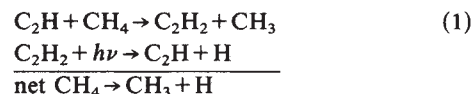
on micrometre-sized nickel grains at 500 K can lead to equilibration times for exchange of deuterium between H_2O and H_2 of 3×10^5 yr, which is roughly the dynamic timescale for the saturnian nebula²¹. The dynamic timescale gives a crude upper limit to the lifetime of material in the saturnian nebula; hence, equilibration times longer than 10^5 yr for the exchange reaction are not plausible. This limits the exchange to high temperatures (> 500 K, achievable in the innermost regions of the nebula with gas then being mixed outward to the cooler regions), and the resulting deuterium enrichment in H_2O to at most a factor of 2. We conclude by analogy that an enhancement factor in deuterated methane of 2 is possible in the primordial saturnian nebula out of which Titan formed. This value may be an upper limit because water could dominate the adsorption sites on the grains. Furthermore, once accretion had started the rates of these reactions would have slowed dramatically because the metallic grains would combine chemically to form minerals with a markedly lower catalytic ability²⁴.

Geiss and Reeves²⁸ examined a variety of mechanisms to explain the deuterium enrichment in terrestrial planets and meteorites, and concluded that exchange mechanisms in the solar nebula were probably less important than earlier enhancement processes in interstellar clouds out of which the solar nebula presumably formed. It is possible that some interstellar grains, greatly enriched in deuterium, retained the enrichment during heating processes accompanying the formation of the solar nebula and/or passage through the accretion shock at the solar nebula/interstellar medium boundary. However, such grains, accreting onto Titan during or shortly after its formation could have been subjected to temperatures in excess of 500 K for perhaps as long as 10^8 yr after the accretion of Titan, based on detailed models by one of us (J.I.L.)³⁶. If the highly deuterated grain material was in thermodynamic contact with a substantial reservoir of non-enriched hydrogen in the primordial atmosphere during this time, dilution of the enrichment could have occurred. This possibility is admittedly very speculative and requires modelling beyond the scope of the present effort. Exchange reactions in the Saturn nebula, therefore, probably

**Fig. 1** Enrichment factor E as defined in the text versus R , the ratio of total initial CH_4 reservoir to the total present CH_4 reservoir.

produced at most a factor of 2 deuterium enrichment in methane incorporated into Titan from the nebular gas; we acknowledge, however, that highly deuterated material in interstellar grains could have been incorporated in Titan, avoided reprocessing and possibly increased the enhancement of deuterated methane.

In the atmosphere of Titan, CH_4 is the major observed hydrogen reservoir. Recent photochemical modelling^{29,30} suggests that a large quantity of CH_4 has been converted into heavier hydrocarbons. The destruction of CH_4 occurs mainly through the catalytic cycle



Following the escape of hydrogen from Titan's atmosphere, CH_3 radicals combine irreversibly to form C_2H_6 . Subsequent reactions lead to the production of smaller amounts of other hydrocarbons. The current rate of destruction of CH_4 (referred to the surface) is $\phi = 1.5 \times 10^{10} \text{ cm}^{-2} \text{ s}^{-1}$. Rate coefficients for reactions of deuterated molecules are usually much smaller than the corresponding value for the molecule composed of the lighter isotope³¹⁻³⁴. The rate coefficient for



has not been measured. Because of the greater binding energy of D compared with H, the rate coefficient for reaction (2) may only be a fraction of that for (reaction 1), that is, $k_2 = rk_1$ where $r < 1$. We adopt two values for r , 0.75 (case A) and 0.875 (case B), the former being the preferred value (A. H. Laufer, personal communication). We assume that reactions (1) and (2) are the primary cause of isotopic enrichment in the photochemical model.

The theory of isotopic enrichment in a planetary atmosphere due to photochemical processes has been worked out by McElroy and Yung³⁵, whose methodology and notation we follow. Let $N_1(t)$ and $N_2(t)$ be the total column abundance of CH_4 and CH_3D , respectively, on Titan at time t . The evolution of $N_1(t)$ and $N_2(t)$ is given by

$$\frac{dN_1(t)}{dt} = -\phi, \quad \frac{dN_2(t)}{dt} = -rf(t)\phi, \quad f(t) = \frac{N_2(t)}{N_1(t)}$$

where ϕ is the column integrated destruction rate of CH_4 (assumed to be constant with time) defined earlier, $f(t)$ is CH_3D to CH_4 ratio, and time is defined so that $t = 0$ refers to the time of formation of Titan and $t = \tau = 4.5 \times 10^9$ yr corresponds to the present. We solve this set of equations by using the observed present value $f(\tau)$ and integrating the equations backwards to $t = 0$ for a range of assumptions for $N_1(t)$. The most interesting results in this computation are the ratio of the initial reservoir

Table 2 Enrichment factors for deuterium on Titan

Process	Enrichment factor	Remarks
(1) Cloud formation	1	See text
(2) C ₂ H ₆ -CH ₄ ocean	1-1.3	Assuming 20% CH ₄ , 80% C ₂ H ₆ ocean
(3) Ocean-ice crust formation	1	See text
(4) Equilibrium between CH ₄ and H ₂ in the saturnian nebula	1-2	Assuming 500 K nebula temperature; isotopic exchange catalysed by metal grains
(5) Atmospheric evolution	1.7	Based on curve A in Fig. 1 and $R = 10$
Total	1.7-4.4	Products of all enrichment factors (1)-(5)

of CH₄ to the present reservoir which is $R = N_1(0)/N_1(\tau)$, and the enrichment factor due to atmospheric evolution which is $E = f(\tau)/f(0)$.

A simple analytical solution exists, which relates E and R , $E = R^{1-\tau}$. The numerical results are summarized in Fig. 1. The enrichment factor E is plotted against R , which is a measure of the extent of atmospheric evolution. For both case A ($r = 0.75$) and case B ($r = 0.875$), E increases with R , and could become very large if $R \gg 100$. For an ocean composition of 20% CH₄ and 80% C₂H₆ (ref. 13), the value for R is 9 and the enrichment factor based on our preferred case A is 1.7. It can be shown that isotopic exchanges such as $H + CH_3D \rightleftharpoons HD + CH_3$ and recombination reactions such as $H + CH_2D + M \rightarrow CH_3D + M$ are of relatively minor importance on Titan. Isotopic re-equilibration could also occur either in the ocean or deeper in Titan's interior. However, temperatures in the ocean are much too low (~ 90 K) to permit appreciable thermal decomposition of either CH₄ or C₂H₆ to form radicals which mediate the isotopic exchange. In addition, since the reactions involve neutral species in an ocean of non-polar molecules CH₄ and C₂H₆, we also would not expect solvent interactions to affect reaction rates. While any treatment of chemical processes in the interior of Titan is necessarily speculative, we would not expect significant re-equilibration to occur at greater depths. This inference is based on current theories of the internal structure of Titan, which propose an icy mantle with a maximum temperature at the core-mantle boundary of ≤ 400 K. The first condition implies greatly restricted mobility of reactants and the second, that temperatures are still too low for thermally-driven reactions to occur.

The relative abundance of deuterium to hydrogen in a planetary atmosphere is one readily available measure of the origin and evolution of the hydrogen bearing volatiles on the planet (see Table 1). If we take the de Bergh *et al.*² value 6×10^{-4} for the ratio CH₃D/CH₄ on Titan, and the Voyager value of 8.7×10^{-5} for Saturn, we note that deuterated methane is enriched 6.9-fold on Titan. The uncertainty associated with the derivation of this number is about a factor of 2-3. Note that the uncertainty must include not only the errors in measurement, but also the plausible range in the fractionation factor between CH₃D and HD within the atmosphere of Saturn²⁰, since the ratio of interest refers to the bulk saturnian value. The physical and chemical enrichment factors considered in this paper are summarized in Table 2. The cumulative enrichment factor is 2-4, which is below the observed value but within the estimated uncertainties of the observations. It is more difficult to interpret the observed value of CH₃D/CH₄ obtained by Kim and Caldwell¹. We have neglected early evolution of Titan's atmosphere driven by planetesimal impacts and early solar extreme ultraviolet which could have increased the atmospheric D/H ratio, allowed some re-equilibration between deuterated species, or

provided an era of enhanced photodissociation of methane. Future observations of deuterated species on Titan and the giant planets are obviously needed to remove the large uncertainties in the D/H measurements. For a more realistic treatment of the photochemically-driven evolution of D/H on Titan, we recommend a laboratory experiment to measure the relative rate constants of the reaction C₂H with CH₃D and CH₄.

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- Kim, S.-J. & Caldwell, J. *Icarus* **52**, 473-482 (1982).
- de Bergh, C. *et al. Bull. Am. astr. Soc.* **13**, 703 (1981).
- Hanel, R. *et al. Science* **212**, 192-200 (1981).
- Kunde, V. *et al. Astrophys. J.* **263**, 443-467 (1982).
- Courtin, R. *et al. Astrophys. J.* **287**, 899-916 (1984).
- McElroy, M. B., Prather, M. J. & Rodriguez, J. M. *Science* **215**, 1614-1615 (1982).
- Donahue, T. M. *et al. Science* **216**, 630-633 (1982).
- Craig, H. *Science* **133**, 1833-1834 (1961).
- Craig, H. & Lupton, J. E. *Earth planet. Sci. Lett.* **31**, 369-385 (1976).
- Fallick, A. E. *et al. Lunar planet. Sci. Abstr.* **XIV**, 183-184 (1983).
- Bjoraker, G. L. thesis, Univ. Arizona (1985).
- Armstrong, G. T., Brickwedde, F. G. & Scott, R. B. *J. Res. natn. Bur. Stand.* **55**, 39-52 (1955).
- Lunine, J. I., Stevenson, D. J. & Yung, Y. L. *Science* **222**, 1229-1230 (1983).
- Miller, R. C. & Staveley, L. A. K. *Adv. Cryog. Eng.* **21**, 493-500 (1976).
- Cheung, H. & Wang, D. I.-J. *J. E. C. Fund.* **3**, 355-361 (1964).
- Parrish, W. R. & Hiza, M. J. *Adv. Cryog. Eng.* **21**, 485-492 (1976).
- Lunine, J. I. & Stevenson, D. J. in *Proc. NATO Conf. Ices in the Solar System* (ed. Klinger, J.) (Reidel, Dordrecht, 1985).
- Lunine, J. I. & Stevenson, D. J. *Astrophys. J. Suppl. Ser.* (in the press).
- Hubbard, W. B. & MacFarlane, J. J. *Icarus* **44**, 676-682 (1980).
- Beer, R. & Taylor, F. W. *Astrophys. J.* **179**, 309-327 (1973).
- Stevenson, D. J. *Planet. Space Sci.* **30**, 755-764 (1982).
- Prinn, R. G. & Fegley, B. Jr *Astrophys. J.* **249**, 308-317 (1981).
- Solomon, P. M. & Woolf, N. J. *Astrophys. J. Lett.* **180**, L89-L92 (1973).
- Anders, E., Hayatsu, R. & Studier, M. H. *Science* **182**, 781-790 (1973).
- Lewis, J. S. & Prinn, R. G. *Astrophys. J.* **238**, 357-364 (1980).
- McKee, D. W. & Norton, F. J. *J. phys. Chem.* **68**, 481-489 (1964).
- McKee, D. W. *J. phys. Chem.* **70**, 525-530 (1966).
- Geiss, J. & Reeves, H. *Astr. Astrophys.* **93**, 189-199 (1981).
- Strobel, D. F. *Icarus* **21**, 466-470 (1974).
- Yung, Y. L., Allen, M. & Pinto, J. P. *Astrophys. J. Suppl. Ser.* **55**, 465-506 (1984).
- Wiberg, K. B. & Motell, E. L. *Tetrahedron* **19**, 2009-2023 (1963).
- Husain, D. *et al. JCS Faraday Trans. II*, **80**, 713-728 (1984).
- Pasteris, L. *et al. Ber. Buns. Phys. Chem.* **88**, 568-571 (1984).
- Tschuikow-Roux, E. & Niedzielski, J. *J. Photochem.* **27**, 141-150 (1984).
- McElroy, M. B. & Yung, Y. L. *Planet. Space Sci.* **24**, 1107-1113 (1976).
- Lunine, J. I. thesis, California Institute of Technology (1985).

Antarctic and non-Antarctic meteorites form different populations

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Compared with non-Antarctic falls, Antarctic meteorites include many of hitherto rare, unique or unknown type¹, such as lunar and putative martian samples²⁻⁴. Attention has not previously been paid to the more common sorts of Antarctic meteorites and here we focus on the most frequently encountered, H5 chondrites. We find that compositional differences between Antarctic and non-Antarctic specimens of this class, especially those involving thermally sensitive trace and ultra-trace elements, are so substantial that it seems extremely doubtful that the two sample populations are derived from the same parent population. This implies that

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the contemporary falls (less than 200 years old) and the substantially older (on average, 300,000 years for Victoria Land) Antarctic meteorites either differ in genetic history or sample different extraterrestrial parent populations, or both.

At present, over 7,000 meteorites have been recovered from opposite ends of Antarctica, mainly from Queen Maud Land⁵, and from Victoria Land in East Antarctica and Marie Byrd Land in West Antarctica⁶. Some samples can confidently be shown to be fragments of the same meteorite, while others are unique. Taking the estimate that Antarctic meteorite impacts yielded, on average, 2–6 fragments per impact⁷, the recovered meteorite population represents 1,200–3,500 separate events. This is comparable to the 2,611 meteorites⁸ recovered elsewhere on the Earth during the past few centuries, despite the fact that the Antarctic sample essentially represents only 15 years of collection.

The contents of selected trace elements in Antarctic and non-Antarctic congeners of rare type often differ, seemingly reflecting actual source variations^{9–13} rather than weathering of Antarctic meteorites since their arrival on Earth (typically $\geq 10^5$ yr⁻¹). These differences for uncommon meteorites suggested to us that common meteorites might also show inherent differences. Indeed, merely considering the grossest classification (Table 1), the Antarctic Search for Meteorites (ANSMET) sample contains proportionally fewer stony-irons and irons than do non-Antarctic falls. Ordinary chondrites (H, L, LL for high, low or very low total Fe contents) from Victoria Land (Table 1) and Queen Maud Land¹⁴ also differ strikingly: LL chondrites are deficient and the H/L chondrite ratio is triple the value for non-Antarctic falls (and finds)⁸.

To look for differences on a finer scale, we analysed H5 chondrites for trace elements (Co, Au, Sb, Se, Rb, Cs, Te, Bi, Ag, In, Tl, Zn and Cd) that vary widely in meteorites and yield important genetic information. These elements represent a variety of geochemical behaviours and span broad volatility and/or mobility ranges. This and their trace concentration (levels of parts per million (p.p.m.) to parts per trillion (p.p.t.)), which amplify small absolute variations into large relative ones, make these elements especially useful for this study. We chose H chondrites not only because they form the majority of Antarctic meteorites but also because concentrations of many trace elements in non-Antarctic L4–6 chondrites are reduced by extended heating from collisional break-up of their parent body 5×10^8 – 6×10^8 yr ago^{15–17}. Hence, for a complete comparison, shock histories of Antarctic L chondrites must also be determined, a time-consuming task. (Preliminary data indicate that, on average, ordinary chondrites from Antarctica differ in shock histories from their non-Antarctic brethren.) In non-Antarctic H4–6 chondrites, volatile/mobile trace-element contents reflect early heating¹⁸, making later shock history irrelevant. (This trace-element difference parallels major element and thermoluminescence trends^{16,19}.) Although mobile trace-element contents vary little with petrological type in type 4–6 L and H chondrites^{15–18}, we minimized variables by studying type 5, the most common sort of H chondrite. Since Queen Maud Land and Victoria Land meteorites differ in mean terrestrial age¹, we studied samples only from the latter and non-Antarctic falls. The meteorites were analysed by neutron activation analysis with radiochemical separation.

To assess accuracy and experimental precision, we analysed Allende standard reference meteorite powder before beginning the actual study. Allende results of analysts whose H5 chondrite data are involved in this study are listed in Table 2; none differs significantly from previous data (Student's *t*-test at the 5% level), other than Co which differs at the 10% test level. With 13 elements, this seems fortuitous and we do not suspect systematic analytical bias. From these homogeneous replicates, we estimate relative experimental precisions of 4–10% for all elements. Replicate analyses of 0.2–0.3-g chips of the same ordinary chondrite^{15–17} reveal slightly poorer but comparable precision. This

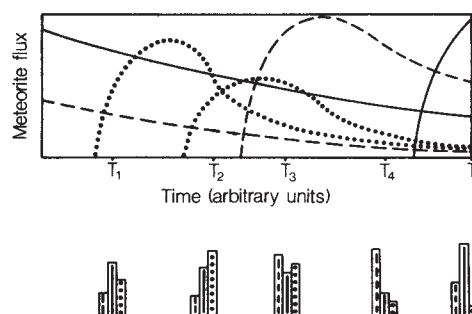


Fig. 1 Temporal variation of meteorite flux. Three sources are represented. Sources A (solid lines) and B (dashed lines) each represent a burst of meteoritic material ejected from an extraterrestrial source by a large impact in the same parent region that produced the smoothly decaying flux, generated by continuous impacts decreasing in frequency with time. Source C (dotted lines) represents meteoritic material ejected from a source region in bursts from two major impacts. Relative proportions at five different times are indicated by histograms at the bottom; these differ with time.

should not affect our conclusions here because all samples studied were chips of comparable mass and experimental uncertainties should cloud, not simulate, compositional differences. Even severe Antarctic weathering has only a minor role in perturbing trace-element contents in H5 chondrites: Bi and Cs are significantly higher and Co, Sb and Tl possibly so in types A and B samples than in severely (C) weathered ones. We have thus calculated means for these elements using only samples of weathering types A and B.

Most statistical tests implicitly assume that sample populations follow either normal or log-normal gaussian compositional distributions²⁰. Our Co, Au, Se and Zn data follow a normal gaussian better than a log-normal one while Sb, Rb, Cs, Te, Ag, In, Bi, Tl and Cd better follow a log-normal distribution. The choice of a particular distribution may slightly decrease or increase the strength of a given difference, but should not affect overall trends or conclusions.

Eight of 13 elements compared in Antarctic and non-Antarctic samples differ using a single-tailed *t*-test (Table 3); we consider differences at the 5% level to be significant, those at the 10% level as possibly so. (If the eight weathering-type C chondrites are included, Sb and Tl differences become possibly significant; the Bi difference is no longer statistically significant. Hence, 7 of 13 elements differ—still an impressive proportion. Essentially the same differences hold if the Antarctic population is divided into two randomly chosen, equal subsets and compared with the non-Antarctic population.) These data provide strong reason to doubt that both sample populations were drawn from the same parent population. If this is so, the simplest and most direct conclusion is that H5 chondrites that fell in Victoria Land 1×10^5 – 7×10^5 (average 3×10^5) yr ago include a substantial

Table 1 Comparative numbers of selected meteorite types found in Victoria Land and falling in non-Antarctic regions

Meteorite type	Victoria Land*		Non-Antarctic ⁸	
	No.	%	No.	%
Chondrites	756	92.4	784	86.6
{ H	542	66.3	276	30.5
{ L	167	20.4	319	35.2
{ LL	24	2.9	66	7.3
Achondrites	45	5.5	69	7.6
Irons	14	1.7	42	4.6
Stony-Irons	3	0.4	10	1.1
Total	818	100.0	905	100.0

* Data corrected for known pairing. More work needs to be done on pairing in equilibrated ordinary chondrites.

component deriving from parent regions different from those yielding contemporary (0–200-yr-old) non-Antarctic falls.

It is unlikely that these compositional differences are related to Antarctic weathering, since the compositions of Antarctic and non-Antarctic meteorites of rare types are strongly correlated^{10,11}, there is no evidence in Antarctic H5 chondrite interiors for loss of trace elements known to be leached from meteorite exteriors during Antarctic weathering⁹ and no significant compositional difference exists between H5 chondrite interiors and weathering rinds beyond that attributable to chance. Furthermore, differences in direction of trace-element enrichments exist in H5 and L6 chondrites²¹ and, in general, variances for Antarctic chondrite distributions are lower than for non-Antarctic ones (see Table 3), probably because of shock-induced trace-element loss; weathering should broaden, not narrow, innate distributions²¹.

The *t*-test implicitly assumes equal population variances, but the Levene test²² for elements in Table 3 reveals that Se, Rb, Zn and Cd variances in Antarctic and non-Antarctic sample populations differ at the 10% test level. However, for 13 elements, 1 or 2 may differ at the 10% test level purely by chance. The Welch or Brown-Forsythe test for populations with unequal variances²² indicates virtually no change in significance levels for differences in means. This subtlety may be ignored in comparing sample populations.

Table 2 Trace-element data for homogenized Allende standard reference meteorite powder

Element	This work*		Purdue mean*†	Ref. 26*‡
	D.W.L.	J.E.D.		
Ag (p.p.b.)	105 ± 8 (5)	95.2 ± 9.5 (6)	98.4 ± 9.3 (53)	
As (p.p.m.)	1.62 ± 0.11 (5)		1.50 ± 0.21 (27)	
Au (p.p.b.)		145 ± 14 (5)	145 ± 10 (16)	
Bi (p.p.b.)	49.6 ± 3.5 (7)	44.9 ± 4.2 (5)	48.6 ± 3.3 (36)	
Cd (p.p.b.)	525 ± 47 (14)	463 ± 45 (6)	508 ± 58 (50)	
Co (p.p.m.)	554 ± 53 (10)	665 ± 37 (6)	612 ± 44 (60)	600 ± 100
Cs (p.p.b.)	86.2 ± 7.5 (8)	87.1 ± 3.8 (6)	87.2 ± 5.2 (54)	100
In (p.p.b.)	29.4 ± 3.6 (11)	29.6 ± 2.9 (6)	30.7 ± 3.3 (46)	29 ± 1
Rb (p.p.m.)	1.24 ± 0.15 (4)	1.08 ± 0.07 (6)	1.11 ± 0.14 (48)	1.2 ± 0.1
Sb (p.p.b.)	89.8 ± 4.2 (5)	92.6 ± 4.2 (5)	84.0 ± 13.8 (33)	
Se (p.p.m.)	8.83 ± 0.70 (5)	9.19 ± 0.38 (6)	8.77 ± 1.17 (38)	
Te (p.p.m.)	1.02 ± 0.09 (14)	0.95 ± 0.08 (6)	1.02 ± 0.10 (59)	
Tl (p.p.b.)	62.2 ± 5.3 (7)	59.7 ± 6.8 (6)	61.2 ± 4.5 (62)	
Zn (p.p.m.)	119 ± 9 (11)	111 ± 11 (6)	116 ± 8 (59)	110 ± 5

* Uncertainties are one sample standard deviation; numbers in parentheses are number of replicates. p.p.b., parts per 10⁹.

† All previous analyses were performed at Purdue (see ref. 25 and references therein).

‡ Recommended values in ref. 26 are from far fewer data than those included in the Purdue mean.

A conceivable source of systematic bias is that different analysts in our group measured non-Antarctic and Antarctic H5 chondrites. Hence, we normalized the data of an analyst (Table 2) to the Allende mean determined by that analyst and used these ratios to assess sample population compositional differences. This implicitly treats analytical bias as multiplicative rather than additive, a gross overcorrection. Even with this over-conservative approach, 8 of 13 elements differ, Sb, Tl and Zn being possibly significant (10% level) and Co, Se, In, Bi and Cd differing at the 5% level. The non-Antarctic H4–6 chondrites we studied have lowered K/Ar age¹⁸. As radiogenic gas data are rare for Antarctic samples, this could have introduced a sample selection bias. However, when the 3 H5 chondrites with K/Ar ages ≤ 3.1 Gyr in the non-Antarctic population are ignored, 8 of 13 elements still differ, 6 at the 5% confidence level (Table 3). Hence, such bias cannot explain the compositional differences between the two populations. Finally, we used the Mann–Whitney non-parametric test to compare Antarctic and non-Antarctic sample populations²³. Once again, differences are common (Sb, Se, Rb, Bi, In, Tl and Cd being significant and Zn possibly so); therefore, elemental distribution modelling is not biasing our results.

Having eliminated these possibilities, we conclude that only extraterrestrial causes can account for the observed compositional variations between Antarctic and non-Antarctic congeners. Possibilities include differences in sample population physical properties, orbital characteristics and meteoroid flux with time; each of these is problematic.

Any difference in physical properties (such as crushing strength) that might generate a difference between the contemporary flux and the Antarctic population would imply a chemical subdivision of H5 chondrites. Qualitatively, the unusual nature of the numerous Antarctic meteorites could be accounted for if they are derived from parent bodies in highly inclined orbits. However, at 1 AU, this could account for a fall frequency enhancement of only a few per cent and would also imply fundamentally different parent regions.

As the mean terrestrial ages of the two groups differ by about 10⁵ yr¹, a change in meteoroid flux with time could be invoked. Figure 1 illustrates a change in the relative proportions of three sorts of samples, which represent different meteorite types or different parent body regions of the same meteorite type. Differences in number and kind of meteorites of rare or unique type in non-Antarctic falls and Antarctic finds^{1–4} illustrate the former, differences among H5 chondrites (Table 3), the latter. However, current dynamic models imply chondritic flux averaging over their 10⁶–10⁷-yr cosmic-ray exposure age timescale²⁴.

Table 3 Comparison of significant differences in mean trace-element contents in Antarctic and non-Antarctic H5 chondrites

Element	Antarctic*	All	Non-Antarctic*		Significance level
			Significance level	>40% ⁴⁰ Ar retention	
Sb (p.p.b.)	83 ⁺²⁴ ₋₁₉ (14)†	69 ⁺²¹ ₋₁₆ (18)	>97	69 ⁺²² ₋₁₆ (16)	>90
Se (p.p.m.)	9.0 ± 1.1 (22)	8.2 ± 1.2 (20)	>99	8.1 ± 1.0 (17)	>99
Rb (p.p.m.)	2.0 ^{+0.5} _{-0.4} (22)	2.5 ^{+1.8} _{-1.0} (20)	>97	2.5 ^{+1.9} _{-1.1} (17)	>95
Bi (p.p.b.)	2.8 ^{+7.1} _{-2.0} (14)†	1.14 ^{+2.4} _{-0.37} (20)	>98	1.05 ^{+2.3} _{-0.72} (17)	>99
In (p.p.b.)	0.21 ^{+0.78} _{-0.16} (22)	0.49 ^{+1.5} _{-0.37} (20)	>97	0.57 ^{+1.8} _{-0.43} (17)	>99
Tl (p.p.b.)	0.81 ^{+2.7} _{-0.62} (14)†	0.24 ^{+1.7} _{-0.21} (20)	>96	0.28 ^{+2.1} _{-0.25} (17)	>90
Zn (p.p.m.)	42.8 ± 8.8 (22)	53.1 ± 24.0 (20)	>96	53.3 ± 25.1 (17)	>96
Cd (p.p.b.)	0.72 ^{+2.0} _{-0.53} (23)	3.7 ⁺¹⁶ _{-3.0} (20)	>99	3.8 ⁺¹³ _{-2.8} (17)	>99

Samples weighed 0.2–0.3 g. Potentially contaminated surfaces were removed from all samples, which were analysed as chips. All elements were measured in each sample to minimize heterogeneity effects. To exclude paired samples (that is, different specimens from the same Antarctic meteorite impact), we selected H5 chondrites from widely dispersed parts of Victoria Land whenever possible. When selecting samples from nearby regions, we used field data and/or contents of cosmogenic ²⁶Al to choose unpaired ones. We omitted regolith breccias from consideration since compositionally they reflect peculiar individual histories²⁷. To minimize systematic analytical bias, we ignored compositional data by others for non-Antarctic H5 chondrites (no previous Antarctic results exist). Elsewhere, we report our H4–6 chondrite data and compare these with others; agreement is as usual^{15–17}.

* Uncertainties listed are 1σ values for normal (Se, Zn) or log-normal distributions. Numbers in each sample population are listed in parentheses.

† Mean for weathering types A and B only. Raw data can be obtained from the authors.

While a gross error in the dynamic arguments may be unlikely, this possibility should be checked in the absence of a viable alternative explanation.

If there are compositional differences between Victoria Land and non-Antarctic meteorites, they should not be confined to rare or unique types and H5 chondrites; indeed, H4-6 and L4-6 chondrites differ compositionally²¹. Whether Queen Maud Land meteorites constitute a subpopulation is a possibility we are now investigating.

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1. Bull, C. B. & Lipschutz, M. E. *Lunar planet. Inst. (Houston) tech. Rep.* **82-03** (1982).
2. Marvin, U. B. *Geophys. Res. Lett.* **10**, 775 (1983).
3. Yanai, K., Kojima, H. & Katsushima, T. *Meteoritics* **19**, 342-343 (1984).
4. Bogard, D. D., Nyquist, L. E. & Johnson, P. *Geochim. cosmochim. Acta* **48**, 1723-1739 (1984).

5. Yanai, K. & Kojima, H. *Proc. 9th Symp. Antarctic Meteorites* (ed. Nagata, T.) 18-34 (National Institute of Polar Research, Tokyo, 1984).
6. Antarctic Meteorite Working Group *Antarctic Meteorite News*. NASA Johnson Space Center, Houston, (1978-85).
7. Scott, E. R. D. *Proc. 9th Symp. Antarctic Meteorites* (ed. Nagata, T.) 102-125 (National Institute of Polar Research, Tokyo, 1985).
8. Graham, A. L., Bevan, A. W. R. & Hutchison, R. *Catalogue of Meteorites* 4th edn (British Museum (Natural History), London, 1985).
9. Biswas, S., Ngo, H. T. & Lipschutz, M. E. *Z. Naturforsch.* **35a**, 191-196 (1980).
10. McSween, H. Y. Jr *et al. Earth planet. Sci. Lett.* **45**, 275-284 (1979).
11. Biswas, S., Walsh, T. M., Ngo, H. T. & Lipschutz, M. E. *Proc. 6th Symp. Antarctic Meteorites* (ed. Nagata, T.) 221-228 (National Institute of Polar Research, Tokyo, 1981).
12. Verkoeteren, R. M., Dennison, J. E. & Lipschutz, M. E. *Geophys. Res. Lett.* **10**, 821-824 (1983).
13. Smith, M. R. *et al. J. geophys. Res.* **81** Suppl., B216-B630 (1984).
14. Mason, B. & Yanai, K. *Proc. 8th Symp. Antarctic Meteorites* (ed. Nagata, T.) 7-28 (National Institute of Polar Research, Tokyo, 1983).
15. Huston, T. J. & Lipschutz, M. E. *Geochim. cosmochim. Acta* **48**, 1319-1329 (1984).
16. Neal, C. W., Dodd, R. T., Jarosewich, E. & Lipschutz, M. E. *Geochim. cosmochim. Acta* **45**, 891-898 (1981).
17. Walsh, T. M. & Lipschutz, M. E. *Geochim. cosmochim. Acta* **46**, 2491-2500 (1982).
18. Lingner, D. W., Huston, T. J. & Lipschutz, M. E. *Meteoritics* **19**, 261-262 (1984).
19. Sears, D. W. G., Bakhtiar, N., Keck, B. D. & Weeks, K. S. *Geochim. cosmochim. Acta* **48**, 2265-2272 (1984).
20. Sacks, L. *Applied Statistics A Handbook of Techniques* (transl. Regnarowich, Z.) (Springer, New York, 1982).
21. Kaczaral, P. W., Dennison, J. E., Lingner, D. W. & Lipschutz, M. E. *Lunar planet. Sci.* **16**, 418-419 (1985).
22. Dixon, W. J. & Brown, M. B. (eds) *BMDP Biomedical Computer Programs, P-Ser.* (University of California Press, Berkeley, 1979).
23. Neter, J., Wasserman, W. & Whitmore, G. A. *Appl. Statist.*, 370-376 (Allyn & Bacon, Boston, 1978).
24. Wetherill, G. W. *Ann. Rev. Earth planet. Sci.* **2**, 303-331 (1974).
25. Verkoeteren, R. M. & Lipschutz, M. E. *Geochim. cosmochim. Acta* **47**, 1625-1633 (1983).
26. Jarosewich, E., Clarke, R. S. Jr. & Barrows, J. N. (eds.) preprint, US National Museum (1985).
27. Lipschutz, M. E., Biswas, S. & McSween, H. Y. Jr *Geochim. cosmochim. Acta* **47**, 169-179 (1983).

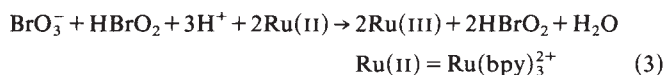
A new optical photochemical memory device in a light-sensitive chemical active medium

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Active media are physical, chemical or biological systems which can compensate their internal energy dissipation by a continuous power input. In homogeneous well stirred chemical systems, self-sustained oscillations can occur. However, in distributed systems which provide spatially distributed energy sources, propagation effects—including waves—can be observed. The most extensively studied example of such a system model is the Belousov-Zhabotinsky reaction^{1,2}. This reaction shows a range of unusual kinetic phenomena, especially in the distributed system, where trigger waves and phase waves can be observed³. By using Ru(bpy)₃²⁺ as catalyst for the Belousov-Zhabotinsky reaction we obtained a light-sensitive chemical active medium and had been able to manipulate the character of wave phenomena by photochemical action⁴. Here we suggest a new kind of optical photochemical memory-device based on photoinduced phase shift in a distributed chemical oscillating medium.

The Belousov-Zhabotinsky reaction is an extremely complex system⁵, but the most important reaction steps can be represented:

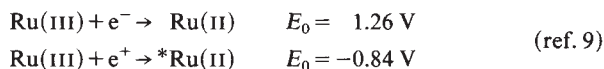


By reducing the mechanism to such simple skeleton the Oregonator⁶ and other models⁷ were developed, which give a satisfying qualitative (and, in some respects, quantitative) account of the complex behaviour of the reaction including the wave phenomena.

For the occurrence of oscillations, the autocatalytic oxidation of the catalyst, reaction (3), is crucial. Bromide ions are the counterpart of the autocatalysis. Via reaction (2) they may inhibit the autocatalytic oxidation. The source for bromide is reaction (4) the reduction of the oxidized catalyst by brominated organic species like bromomalonic acid. For oscillations to occur it is important that the bromide production, reaction (4) is coupled with reaction (3) as a delayed inhibitor production. This means that bromide ions are the most important control intermediate of the system.

In continuous stirred tank reactor (CSTR) experiments we can realize bifurcations between several types of reactions regimes (oscillating to nonoscillating) by varying the bromide input stream. If the system is in the oscillating regime and we chose the bromide concentration is varied only within a narrow range, only the parameters of the oscillations (frequency and amplitude) will be changed⁸.

The light-sensitive Ru-catalysed system offers the possibility of selecting the rate of bromide production by photochemical action. The absorption of light induces a dramatic change in the redox properties of Ru(II).



In contrast to the ground state it is evident that the excited molecule is expected to be a very strong reducing agent. ^{*}Ru(II) in the excited state is able to reduce bromate to the inhibitor bromide directly via:



By illuminating the Ru-catalysed Belousov-Zhabotinsky reaction in the distributed system we can control the propagating effects and manipulate the character of wave phenomena.

In the present work a distributed oscillating system was used. The reaction medium changes colour periodically. Practically, in the absence of stirring, this colour change does not take place

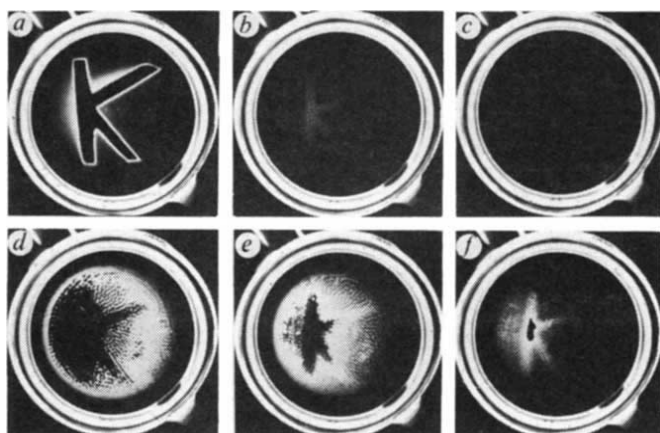


Fig. 1 A new optical photochemical memory-device in a light-sensitive chemical active medium. Initial concentrations were: $\text{NaBrO}_3 = 0.31$; $\text{H}_2\text{SO}_4 = 0.135$; Malonic acid = 0.045; Bromomalonic acid = 0.105; $\text{Ru}(\text{bpy})_3\text{SO}_4 = 0.004$ M. The Petri dish was thermostatted at 25°C . Diameter was 7 cm and depth of the reaction layer 1.3 mm. Illumination was for 15 s from a 25-W tungsten-lamp. Photographs were taken at the following times after illumination through a blue filter: a, 5 s; b, 15 s; c, 20 s; d, 30 s; e, 35 s; f, 45 s.

simultaneously in the whole medium. Spatial inhomogeneities may induce a phase shift in the oscillating reaction. The wall of the reaction vessel is especially a source for such inhomogeneities, due to influence of oxygen. So the oscillating reaction starts at the wall of a Petri dish, moves as a phase wave to the middle point and then vanishes. This phenomenon proceeds with a well defined constant frequency. By taking advantage of the action of light, we can change the conditions spatially; for example, we can induce a phase shift in the illuminating region. This phase shift forms a pattern which is 'frozen' in the oscillating medium and will have reproduced as a positive-negative image several times.

Considering the complex kinetics in the chemical active medium, when contrasted to normal simple chemical or photochemical reactions, new possibilities for storage and processing of information impressed by a photoreaction will be opened. For instance, we may construct a new type of an optical photochemical memory device as is demonstrated in Fig. 1a-f.

In Fig. 1a, the medium is illuminated from above by focusing light through a template in the shape of the letter K. The original state of the medium is coloured. Black areas in the figure correspond to the reduced orange state ($\text{Ru}(\text{II})$) and white areas to the blueish fluorescent oxidized ($\text{Ru}(\text{III})$) state of the system. Under the influence of light (Fig. 1a), the reduced state is stabilized. A phase shift on the oscillating medium between illuminated and nonilluminated areas occurs. In Fig. 1b-f the successive stages in the dark reaction are shown. The medium has stored the photochemically impressed information as a phase shift and can process this information. If we called the first picture (Fig. 1a) a positive image, then after one oscillating cycle this positive is converted into a negative (Fig. 1b). The area that was formerly reduced is now oxidized and the surrounding area is reduced. In an intermediate range, the picture may also be invisible (Fig. 1c). The whole system is in the reduced state.

In Fig. 1d, the letter is in the reduced state and the surrounding area in the oxidized state. In Fig. 1e, the changeover to the reduced state has been completed and a further change to the oxidized state has begun. The change to the oxidized state is hardly complete in Fig. 1f. After some further cycles the contrast will progressively vanish and finally disappear.

This new photochemical system is able to store time-impressed optical information and to process this information in some oscillating cycles, as a dynamically working memory device.

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1. Belousov, B. P. *Sb. Ref. Radiats Med. Moscow* **1958**, 145-147 (1959).
2. Zhabotinsky, A. M. *Dokl. Akad. Nauk SSR* **157**, 392-395 (1964).
3. Zaikin, A. N. & Zhabotinsky, A. M., *Nature* **225**, 535-537 (1970).
4. Kuhnert, L., *Naturwiss.* (submitted).
5. Field, R. J., Körös, E. & Noyes, R. M. *J. Am. Chem. Soc.* **94**, 8649-8664 (1972).
6. Field, R. J. & Noyes, R. M. *J. Chem. Phys.* **60**, 1877-1884 (1974).
7. Rovinsky, A. B. & Zhabotinsky, A. M. *J. Phys. Chem.* **88**, 6081-6084 (1984).
8. De Kepper, P. & Boissonade, J. in *Oscillations and Travelling Waves in Chemical Systems* (eds R. J. Field & M. Burger) (Wiley-Interscience, New York, 1984).
9. Sutin, N. & Creutz, C., *Adv. in Chemistry Series No. 168, Inorganic and Organometallic Photochemistry* 1-27 (1978).

A plate tectonic model of the eastern Indonesia collision zone

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Eastern Indonesia is unique in the present global plate configuration, being situated at the T-junction between the world's two main active orogenic belts—the Alpine-Himalayan and Circum-Pacific belts. Consequently the tectonics of this region must be understood as a zone of interaction between three major plates, whereas most other orogenic areas can be modelled as deformation between just two major plates with numerous intervening microplates or terranes. Previous models to explain the tectonics of eastern Indonesia have generally started from small-scale geological observations and attempted to work upwards to an overall plate synthesis. In this paper I reverse the process, suggesting what might be expected from one particular collision model in a three-plate system and comparing this with what is actually seen in the region's geological record.

Consider a hypothetical three-plate indenter model (Fig. 1a). The body to be indented comprises two plates: a continental plate (A) in the west and an oceanic plate (B) to the east. Continent A is constrained on its western and northern margins, but unconstrained on its other two sides. This continent will be taken as fixed, with other plate movements described relative to it. Oceanic plate B is converging upon continent A, moving due westwards. Convergence between the two is taken up by simple subduction beneath A. The indenter comprises a second continent (C) initially surrounded by passive margins. Continent C is asymmetrical with a large oceanic embayment on its western side. The indenter is required to move northward at a constant speed, but is unconstrained on its sides, and so is free to have an eastward or westward component of movement if the forces acting on it so dictate. The boundary between plates A and C is a simple northward subduction of oceanic plate C below continental plate A. The boundary between plates B and C is a combination of normal subduction of C under B, and sinistral strike-slip parallel to the plate margin taken up within the overriding plate.

The model is arranged so that the indenter collides with the easternmost edge of continent A and the westernmost part of ocean B (Fig. 1b). Upon collision, subduction reversal takes place in the eastern part of the collision zone, with ocean B now subducting beneath continent C. In the western part, continent-continent collision occurs and subduction ceases. Instead a conjugate set of fractures develop in continent A (compare with actual indenter models, for example, in refs 1-3). Because continent A has an unconstrained eastern margin, and because the indenter can have an eastward component of movement, the sinistral set of fractures is preferentially developed. In particular, one fracture originating near the northwest corner of the indenter will take up the bulk of the compensational deformation within continent A, becoming a trench-trench transform⁴

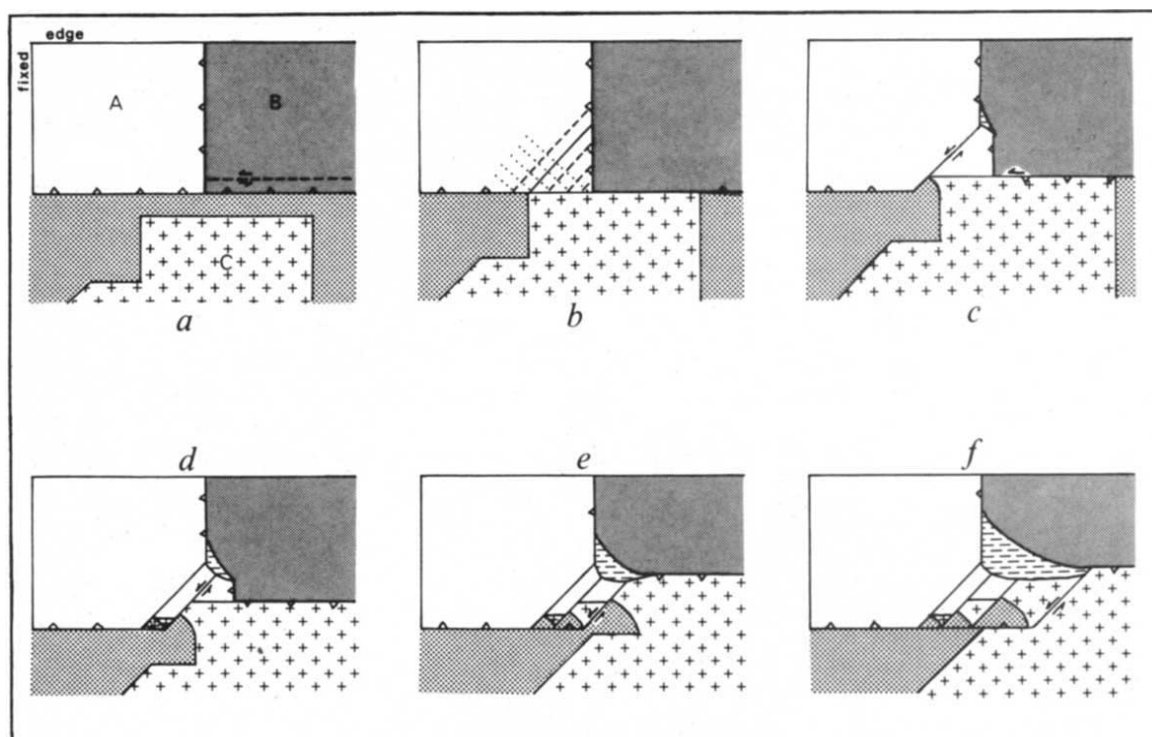
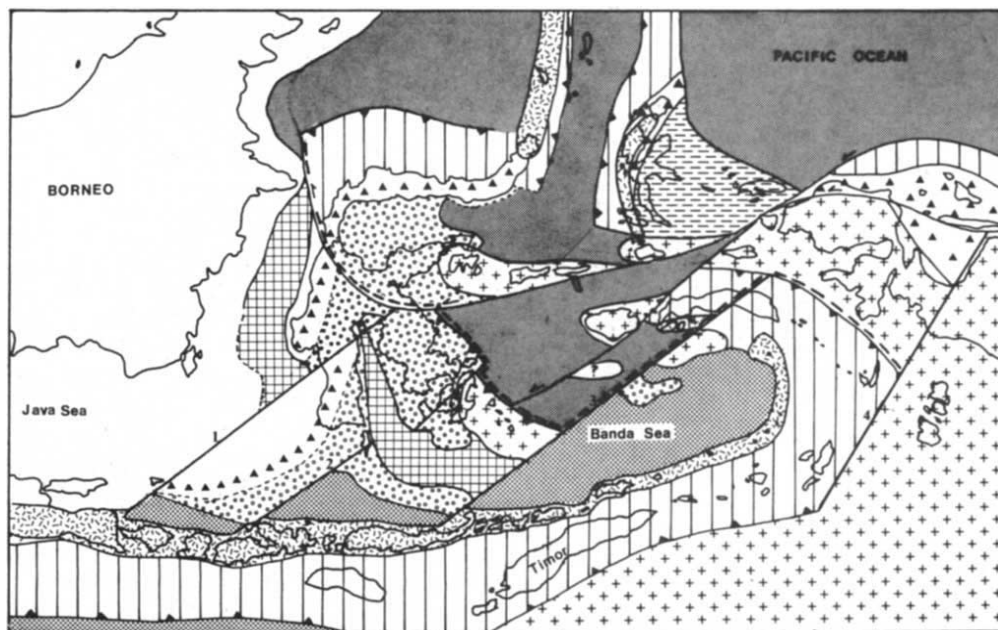


Fig. 1 Hypothetical indentation model to explain the tectonics of eastern Indonesia. *a*, 'Australia' (continent C) moves north to collide with 'Southeast Asia' (continent A). Oceanic areas are stippled. Toothed lines are subduction zones with the teeth on the overriding plate. Half arrows represent strike-slip components. *b*, Upon collision, conjugate fractures develop in Southeast Asia. *c-e*, Development of a series of trench-trench transforms segmenting the former Australia-Asia collision zone. Dashed area is the region of New Guinea-Pacific displaced terranes. *f*, Position immediately prior to second collision of 'Australia' (Timor region) with 'Banda arc'.

Fig. 2 Interpretation of the present configuration of eastern Indonesia in terms of the model developed in the text and in Fig. 1. Crosses: Australian crust. Blank: Asian crust. Dots: Asia-Australia collision zone. Fine stipple: Pacific-related oceanic crust. Heavy stipple: Indian Ocean crust. Cross-hatching: Local seafloor spreading associated with transtension. Irregular dashes: current volcanic arc. Triangles: early Neogene volcanic arc. Toothed lines: subduction zones. Vertical hatching: current forearc. Paired solid and dashed line: southwestern limit of New Guinea-Pacific displaced terranes. Trench-trench transforms are numbered consecutively. Note that division of forearc and Australian crust around the Banda arc is somewhat arbitrary.



(Fig. 1c). This transform is terminated at its northeastern end by a trench-trench-transform triple junction⁴.

At the same time as the trench-trench transform is developing between ocean B and the oceanic part of plate C, the leading edge of the indenter is experiencing a combination of pure and simple shear. The compressive pure shear is produced by the northward movement of the indenter; the simple shear, sinistral in sense, results from the westward movement of plate B and the eastward component of movement of plate C relative to the fixed continent A. The northeastern margin of the indenter thus

experiences transpression^{5,6}, with anticlockwise block rotation⁷ and sinistral displacement of terranes. Consequently, a neck of deformed crust develops between continent A and the indenter, while displaced terranes accumulate in the corner formed by the trench-trench-transform triple junction, hindering the development of the transform (Fig. 1c). Eventually, it becomes easier to initiate a new transform than to rejuvenate the older one. A new transform will be initiated to the southeast of the original one where the neck between the two oceans is narrowest. Once this develops, subduction must commence to the south of

the transform-bounded sliver (Fig. 1d). The pattern of dynamics will then be similar to that after the initial rupture, and after some time the same inhibition of the second transform by displaced terranes will occur, followed by another southward jump to a new transform. However, the time between the first and second jump will be greater than the time from initiation of the original transform to the first jump because the new zone of terrane accretion has first to infill the gap between the rigid edge of continent A and the new triple junction (Fig. 1d).

This repetitive initiation of new transforms after increasingly long periods will continue until the indenter has moved sufficiently far eastward to resume purely northward movement. Progressively larger portions of the oceanic part of plate C will become trapped behind an island arc migrating eastward in steps as successive transforms become inactive (Fig. 1e). Interspersed between these trapped oceanic fragments will be slivers of continental crust derived from both continental masses.

This simple three-plate model can be used as a basis for interpreting the tectonics of eastern Indonesia (Fig. 2). However, the plate convergence vector of the Indo-Australian plate relative to the Southeast Asian plate is not due north, but 20° east of north⁸. Similarly the Pacific plate convergence vector is about 20° north of west. Thus to be more closely comparable with eastern Indonesia, the model outlined above must be rotated 20° clockwise.

If the model is applicable to the tectonic evolution of eastern Indonesia, it implies that the main collision between the Australian and Southeast Asian continents has already taken place. Numerous lines of geological evidence point to a major tectonic reorientation in Southeast Asia during mid Miocene times. This

is here interpreted as the time when indentation was initiated following the main collision orogeny.

The post-collisional offset of Australia relative to Southeast Asia has been taken up on four main transform faults. The first (Fig. 2) accommodated about 300 km offset. The second accommodated about 350 km offset, and the third about 600 km. The sequentially increasing offsets on the transforms explained by the model can be seen from the relative sizes of the portions of Indian Ocean crust trapped in the southern Banda Sea. The eastward younging of volcanic initiation in the Sunda/Banda arc is seen in the decrease in size of the volcanic edifices from west to east.

The final transform may have had more than 400 km of offset. However, with this final transform the simplicity that had previously existed broke down as a result of a second collisional phase (Fig. 1f). The collision of Australian crust with the Banda arc in the vicinity of Timor occurred after the inactivation of the third transform, and after the Banda arc had completed its eastward migration. Analogous indentation processes are now occurring on a smaller scale in the Timor region (T. R. Charlton and A. J. Barber, manuscript in preparation).

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1. Molnar, P. & Tapponnier, P. *Science* **189**, 419–426 (1975).
2. Tapponnier, P. & Molnar, P. *Nature* **264**, 319–324 (1976).
3. Tapponnier, P., Peltzer, G., Le Dain, A. Y., Armijo, R. & Cobbold, P. *Geology* **10**, 611–616 (1982).
4. McKenzie, D. P. & Morgan, W. J. *Nature* **244**, 125–133 (1969).
5. Harland, W. B. *Geol. Mag.* **108**, 27–42 (1971).
6. Sanderson, D. J. & Marchini, W. R. D. *J. struct. Geol.* **6**, 449–458 (1984).
7. McKenzie, D. & Jackson, J. *Earth planet. Sci. Lett.* **65**, 182–202 (1983).
8. Minster, J. B. & Jordan, T. H. *J. geophys. Res.* **83**, 5331–5345 (1978).

Tectonics of the westernmost Gulf of Aden and the Gulf of Tadjoura from submersible observations

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The Gulf of Tadjoura, separating the Danakil and Somalia plates¹, represents the western extension of the Sheba Ridge between Arabia and Somalia in the Gulf of Aden (Fig. 1). It has been assumed that a simple plate-tectonic scheme could be applied to the area with NE–SW transform faults offsetting roughly east–west zones of accretion represented by the major bathymetric troughs^{1–8} (Fig. 1c). More recently this boundary has been interpreted as a westward-propagating rift with its present-day tip on land in the region of the Asal Lake^{9,10}. Here we present data from dives with the submersible *Cyana* which show that there have been two episodes of extensional faulting, an older inactive phase parallel to the general ENE–WSW direction of the gulf and a younger fault set along discontinuous zones striking 140°. There is no surface evidence for any transform fault zone in the area covered and the incipient spreading segments whose geometry implies extension on 050° do not define a plate boundary. The faulting observed is at odds with that predicted by orthodox plate tectonics.

The seismicity recorded locally since 1972 in the Republic of Djibuti shows a marked contrast between a concentrated zone of earthquakes in the Gulf of Tadjoura and Goubbet al Kharab

and a diffuse zone of seismicity west of the Ardoukoba Rift^{6,11} (Fig. 1a). In November and December 1978, a seismic and volcanic event occurred in the western part of the Gulf of Tadjoura with a prominent break marked by a line of aftershocks striking 140° in the area of 42°50' E (Fig. 1b). This fault line remained unexplained in the plate tectonic framework accepted by most investigators (Fig. 1c).

An extensive aeromagnetic survey⁹ of the Republic of Djibuti and the Gulf of Tadjoura up to 44° E shows that the linear, approximately east–west, magnetic anomalies of oceanic character are found only in the eastern part of the Gulf of Tadjoura. West of 43°20' E, the magnetic anomalies are less linear and can often be attributed to point sources. The propagating rift model was partially based on this data set, which used the boundary of the oceanic and continental-like anomalies.

In December 1984, 26 dives were conducted by the French submersible *Cyana* at the axis of the westernmost Gulf of Aden and Gulf of Tadjoura in order to make field observations of a nascent zone of accretion between the Danakil and Somalia plates. The diving programme was designed to scan the geometry of the plate boundary in order to test current plate tectonic models which predict a series of short east–west spreading segments bounded by closely spaced NE–SW transform faults (Fig. 1c). The dives were also designed to complement both previous submersible investigations at the axis of steady-state mid-ocean ridges (12 to 17) and previous extensive land investigations conducted in the Republic of Djibuti around the Gulf of Tadjoura (see ref. 18 for a recent synthesis).

The bathymetry of the western part of the Gulf of Aden and of the Gulf of Tadjoura has been established, following earlier work by Peter and de Wald¹² and Backer *et al.*¹³, using data from the 1977 Sumerouad cruise of the NO *Le Suroit*, and the 1983 Precyad cruise of the NO *Jean Charcot*, which used the Seabeam bathymetric swath system. The maximum depth at the axis of the gulf increases from west to east from 200 m in the Goubbet al Kharab, to 800 m in the Tadjoura Trough (area III in Fig. 2a), to 1,000 m in the Maskali Trough (west portion of

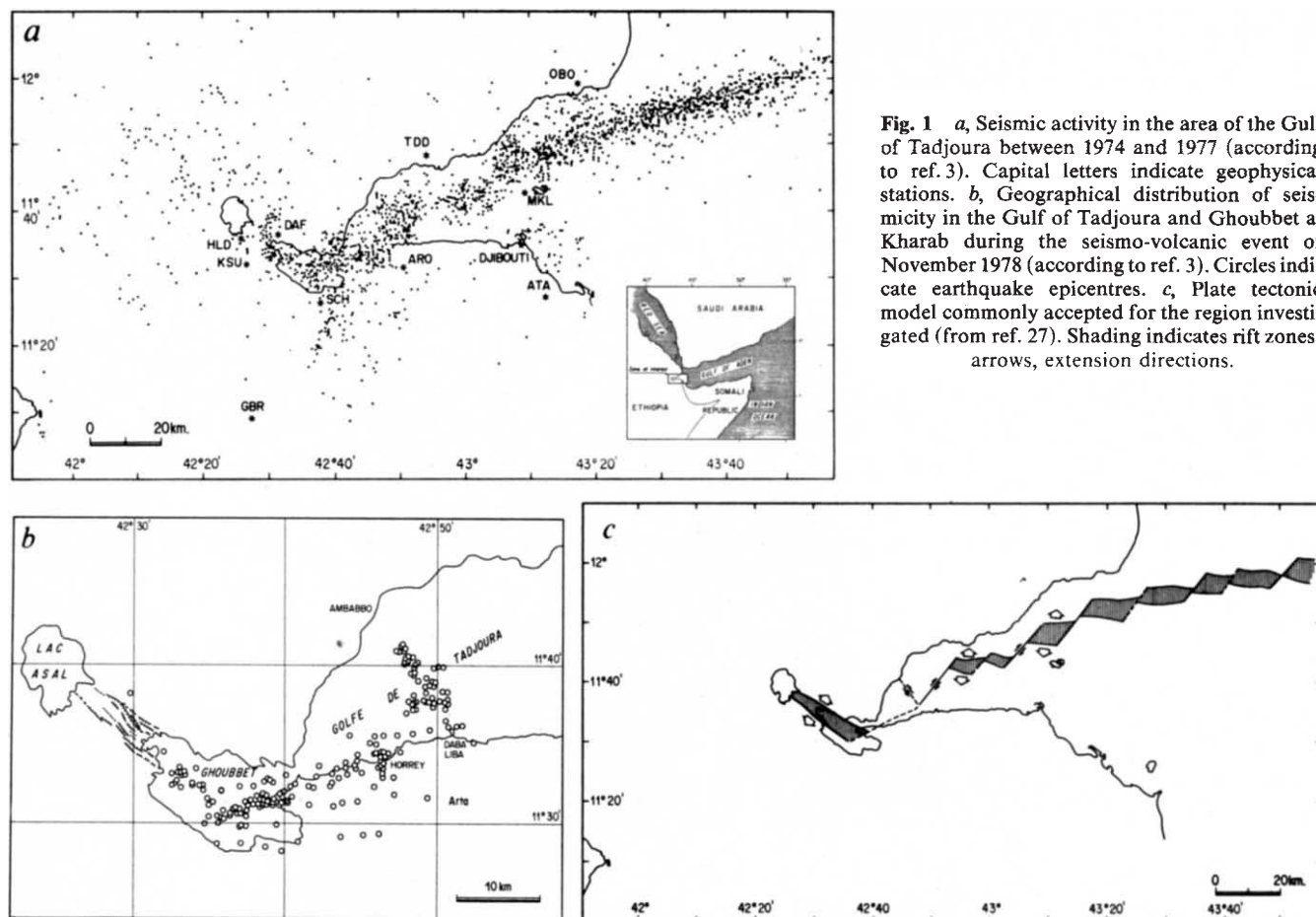


Fig. 1 *a*, Seismic activity in the area of the Gulf of Tadjoura between 1974 and 1977 (according to ref. 3). Capital letters indicate geophysical stations. *b*, Geographical distribution of seismicity in the Gulf of Tadjoura and Ghoubbet al Kharab during the seismo-volcanic event of November 1978 (according to ref. 3). Circles indicate earthquake epicentres. *c*, Plate tectonic model commonly accepted for the region investigated (from ref. 27). Shading indicates rift zones; arrows, extension directions.

area II in Fig. 2*a*). In plan shape the troughs are aligned on a mean 070° direction and are interrupted approximately every 30 km by bathymetric highs (sills) trending NW-SE. Three detailed areas were mapped (zones I, II and III in Fig. 2*a*). The dives were conducted in three additional areas (IV-VI in Fig. 2*a*) to cover the principal supposed transform zones linking the main troughs.

During the *Cyaden* cruise, a total bottom coverage of 118 km was achieved; there were nine dives in zone I (Fig. 2*a*), eight in zone II, four in zone III, two in the area between $42^\circ 50'$ and 43° (zones IV and V) and three in the Ghoubbet al Kharab (zone VI). More than 100 samples were collected *in situ* by the submersible. In addition, three piston cores were taken (one in zone I, one in zone III and one in the Ghoubbet (zone VI)) and two dredge hauls were made (one each in zones I and II). All dive data were positioned using acoustic navigation. The Seabeam maps were constructed using satellite navigation, radar fixes and dead-reckoning. Registration of the Seabeam map with respect to the dive data was made by performing additional bathymetric surveys between the diving periods.

The diving programme has yielded information on volcanism, sedimentation, faulting and hydrothermal activity:

Volcanism. Recent volcanism was found in the two eastern areas and in zone IV. In zone I, the zone of recent flows is very narrow and restricted to a NW-SE 2-km-wide volcanic ridge linking the two main walls of the depression. The flows are glassy pillow lavas sticking out of a heavily sedimented terrane. In zone II, the region of fresh flows is limited to a 5-km-wide NW-SE volcanic ridge. In zone IV, the dive was conducted on a young linear seamount parallel to a NW-SE direction. The older flows are characterized by weathered pillow lavas and occupy the walls of the main depression. Most basaltic samples are aphyric and highly vesicular.

Note that the main bathymetric highs segmenting the depression are to constructional volcanic highs in areas I and II. In areas III and V, the highs correspond to horsts made of corolline (madreporia) material.

Sedimentation. All troughs in the depression are heavily sedimented. A 3.5-kHz seismic survey conducted in zone I showed that the western trough includes at least 60 m of locally faulted hemipelagic sediments, in contrast to the eastern trough which exhibits a quiet regime of sedimentation: the very high rate of sedimentation (100 m Myr^{-1}) determined by Olauson and Olsson¹⁴ in varved sediments nearby $44^\circ 18' \text{ E}$ seems to be confirmed by our observations and particularly by a large amount of sediment cover on parts of volcanic ridges which are known to be recent because they are covered by fresh glass when exposed.

The second type of sediment which has been encountered and sampled during the dives consists of biogenic sediments which are presently being studied to assess vertical movements. **Faulting.** Throughout the study area, two main fault directions are found, 060° and 140° (Fig. 2*c*). In zones I-VI, the active tectonic zones in sediments and recent flows are very localized and along 140° . In zones IV-VI, no 060° faults have been observed. In zones II and III, the 060° faults are clearly older and at present inactive: this is shown by the occurrence of smoothed scarps covered by undisturbed sediment or scarps abutting a flat undisturbed beach-like sediment cover. In contrast, along the 140° fault scarps, reworked sediment and rubble attest to the recent activity. The rim of these fault scarps are generally sharp.

Lastly, in zones I and II, the 060° faults are locally clearly buried by recent lava flows which are cut by the 140° faulting. In these zones, the active 140° faults cut through both lavas and indurated sediments or volcano-sedimentary rocks in which spectacular high pitch striae have been observed on scarps. In

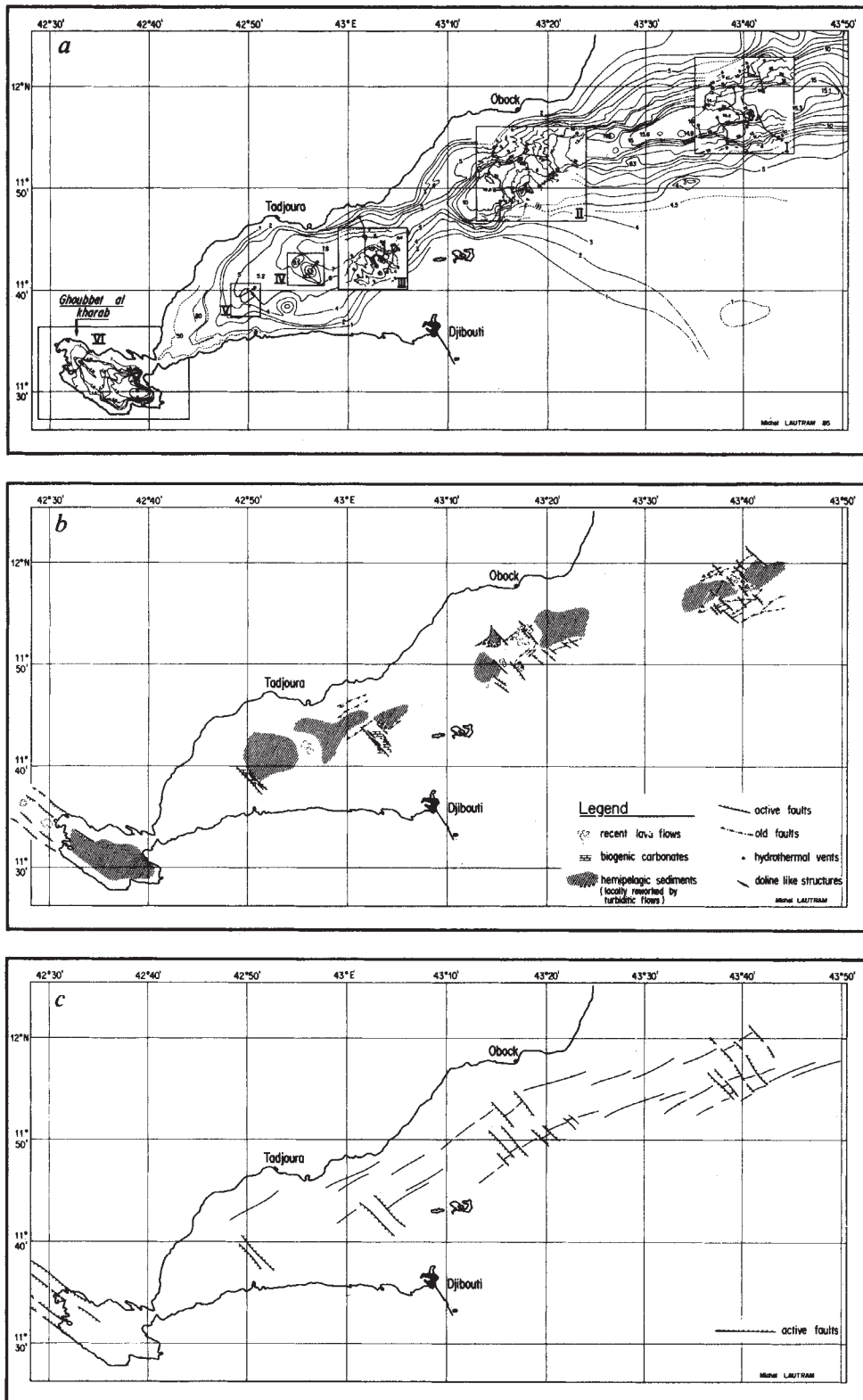


Fig. 2 *a*, Bathymetry of the Gulf of Tadjoura and Ghoubbet al Kharab including details covered by Seabeam (zones I-III). The six dive areas explored during the *Cyaden* programme in December 1984 are marked (contour intervals every 100 m). *b*, Outline of the main geological features directly observed during the *Cyaden* programme. *c*, The two main directions (060° and 140°) of faulting observed in the Gulf of Tadjoura area. Note the absence of NE-SW (010°-020°) faults corresponding to transform direction as proposed by previous tectonic interpretation of this region (to be compared with Fig. 1c).

zone I, however, faulting interference 060 and 140 is pronounced at the outcrop scale and elsewhere. Some 060 faults have been slightly reactivated, as shown by a light-coloured strip of disturbed sediment at the foot of scarps. Low pitch striae were never observed and the vertical component of displacement along the two sets of faults was the only one which could be assessed.

In the western areas (zones III-VI), alignment of doline-like collapse in sediments is common along 140 in active tectonic

zones. A spectacular example of this was recognized and followed in area V along the line of aftershocks during the seismic crisis of 1978³. It is clear from the observations that: (1) a major change has occurred in the direction of extensional faulting from 060 to 140; as the faulting is not synchronous, none of the two trends can represent a possible transform direction; (2) no typical rift topography has been produced; at the map scale, the active 140 volcanic and tectonic zones constituting discrete

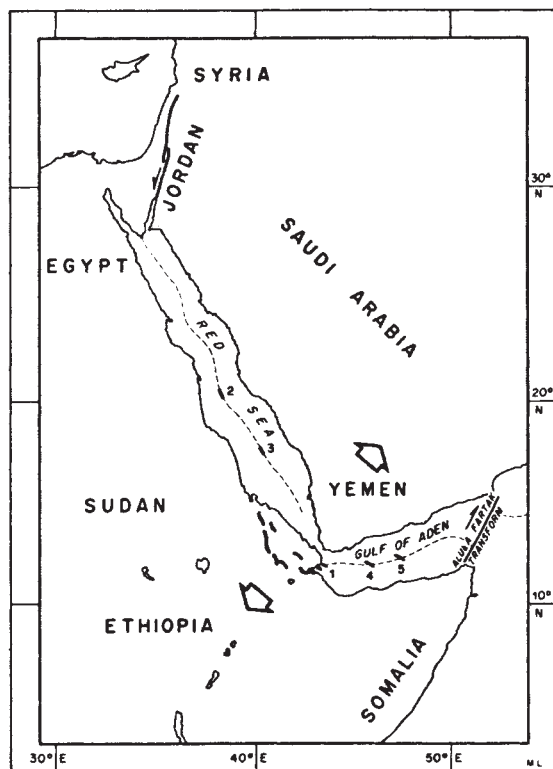


Fig. 3 Active tectonic zones of the Gulf of Aden-Afar-Red Sea-Levant area. The axis of maximum depth of the Gulf of Aden-Gulf of Tadjoura and Red Sea has been marked by a thin dotted line. The zones of recent generation of oceanic (or oceanic-like in the case of Afar) crust have been marked by black lenses with their elongation parallel to that of graben and axial volcanoes. These lenses can be thought of as magma fissures in the crust and lithosphere. The numbered zones of extension represent detailed study areas by Cyaden (zone 1), *Discovery* and *Chain Deeps* (zone 2), Monin *et al.* (zone 3)²⁰, Zonenshain *et al.* (zone 4)¹⁸ and Laughton *et al.* (zone 5)⁷. The entire tectonic system is compatible with rigid motion of Saudi Arabia away from Africa between the transform faults represented by the Alula-Fartak and Levant faults. This motion is indicated by the two wide arrows. In this model, the African Rift system is inactive and the opening of the Gulf of Aden-Red Sea between the transform faults results in only a few discontinuous loci of emplacement of new oceanic crust in a predominantly continental region.

topographic highs are built on the bottom of the main 060 older sedimented trough.

Hydrothermal activity. Three types of hot water venting have been seen: a linear field of diffuse hydroxide gel-producing warm water on the south wall of zone I along 140° trending faults; an isolated vent with concentrated jetting of hot water in a canyon on the north wall of the main depression in area II; and a field of varicoloured patches of hydrothermal material covering sediments, in area III. No evidence of black or white smoker-type vents or any sulphide deposits was found.

It is clear that the widely accepted plate boundary geometry, as presented in Fig. 1c, is not compatible with the field observations. First, the zones of active extension strike normal to the main elongation of the gulf. Second, these zones are discrete and there is no continuous zone of deformation. Third, there is no evidence of transform fault zones. The existence of poorly developed and discrete accreting zones strongly suggests that the present system is in a nascent and non-steady-state mode.

The overall status of faulting shows that there have been at least two successive stages of extensional faulting, running almost at right angles, the first one probably being responsible for the creation of the gulf. The present direction of rifting oblique to the elongation at the gulf appears to be consistent

throughout, both on land (Ardoukoba Rift) and in the gulf. This extension direction also coincides with the fault breaks outlined during the 1978 seismic and volcanic event, which raises some doubt over the strike-slip earthquake first motion solution for the 1978 series of shocks³; the solution was, however, composite and may therefore be incorrect.

The precise timing of the deformation event characterized by north-west normal faults is not known. Nevertheless, one can argue on the basis of an age of 0.9 Myr¹⁵ for the oceanic tholeiites in the Ardoukoba rift and the homogeneity of the faulting direction that the north-west normal faulting has been initiated within the past million years.

The present faulting pattern in the Ardoukoba Rift—Gulf of Tadjoura region is homogeneous and can be extended to a much wider region (Fig. 3). In the Gulf of Aden, the present zones of accretion also strike NW-SE and appear, at least in the eastern half of the gulf, to be offset by well-defined NE-SW, nearly orthogonal transform faults (that is, the Alula-Fartak Transform Fault). The other recent volcanic zones in the Afar Triangle (Manda-Inakir, Manda Harraro, Erta Ale) are also aligned along a 130–140° direction, but there is no evidence for active transform faulting. The only transform fault (or zone) recognized in Afar is located on land on the south side of the Gulf of Tadjoura¹⁶. In the Red Sea, the direction of present rifting is also NW-SE^{17,18}. It has been argued that, locally, transform faults exist at the axis of the gulf showing a NE-SW direction^{16,17}. The overall pattern of the present extension is compatible with slight motion of only two main rigid plates, Arabia and Africa; the 'oceanic' zones of the Red Sea and Gulf of Aden are linked through the 'continental' region of Afar, including the Gulf of Tadjoura. The proposed interaction between only two plates implies negligible opening along the East African Rift. Our model is at variance with other plate tectonic models which involve at least three plates^{1,2,7,8}, even if the observed spreading direction is compatible with that predicted (035°) by Minster and Jordan¹⁹. In our model, the Afar Triangle could represent a diffuse but coherent relay zone between the Aden Rift and the Red Sea Rift. If this is the case, the notion of a propagating rift could be inappropriate for describing the structural evolution of this region. Indeed, the area between the southward-propagating Red Sea Rift and the westward-propagating Aden Rift is theoretically an area of no strain. In this case also, assuming that the lithospheric units are totally rigid, large true accretion in the wedge would fall to zero at the advancing tip. In contrast, the situation described here concerns a zone of distributed strain and motion between two incipient accreting zones. However, this implies that the lithosphere is not completely rigid.

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1. Le Pichon, X. & Francheteau, J. *Tectonophysics* **26**, 369–406 (1978).
2. Barberi, F. & Varet, J. *Bull. geol. Soc. Am.* **88**, 1251–1266 (1977).
3. Lepine, J. C., Ruegg, J. C. & Anis, A. M. *Bull. Soc. géol. Fr.* **22**, 809–816 (1980).
4. Arthaud, F., Choukroune, P. & Robineau, B. *Bull. Soc. géol. Fr.* **22**, 901, 908 (1980).
5. Arthaud, F., Choukroune, P. & Robineau, B. *Bull. Soc. géol. Fr.* **22**, 909–915 (1980).
6. Needham, H. D. *et al. Earth planet. Sci. Lett.* **28**, 439–453 (1976).
7. Laughton, A. S., Whitmarsh, R. N. & Jones, M. T. *Phil. Trans. R. Soc. A* **267**, 227–266 (1970).
8. Girdler, R. W., Brown, C., Moy, D. J. M. & Styles, P. *Phil. Trans. R. Soc. A* **298**, 1–43 (1980).
9. Courtillot, V., Galdeano, A. & Le Mouél, J. L. *Earth planet. Sci. Lett.* **47**, 144–160 (1980).
10. Courtillot, V. *Phys. Earth planet. Inter.* **21**, 343–350 (1980).
11. Stieljes, L., Joron, J. L., Treuil, M. & Varet, J. *Bull. Soc. géol. Fr.* **17**, 851–862 (1976).
12. Peter, G. & de Wald, O. *Bull. geol. Soc. Am.* **80**, 2313–2316 (1969).
13. Backer, H., Clin, N. & Lange, K. *Mar. Geol.* **15**, 309–327 (1973).
14. Olausson, E. & Olson, I. U. *Palaeogr. Palaeoclimatol. Palaeoecol.* **6**, 87–103 (1969).
15. Richard, O. thesis, Univ. Orsay (1979).
16. Lepine, J. C., Richard, O., Ruegg, J. C., Treuil, M. & Varet, J. *Cr. hebdom. Séanc. Acad. Sci., Paris* **283**, 9–12 (1976).
17. Juteau, Th. *et al. Bull. Cent. Rech. expl. Prod. Elf-Aquitaine* **7**, 1, 217–231 (1980).
18. Zonenshain, L. P., Monin, A. S. & Sorokhtin, O. G. *Geotektonika* **2**, 3–22 (1981) (in Russian).
19. Minster, J. B. & Jordan, T. H. *J. geophys. Res.* **83**, 5331–5354 (1978).
20. Monin, A. S. *et al. Deep Sea Res.* **29**, 361–373 (1982).

Formation of cubic structures in microemulsions containing equal volumes of oil and water

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Microemulsions are dispersions of oil and water stabilized by an interfacial film of surfactant and co-surfactant¹. Frequently a short-chain alcohol, such as butanol or pentanol, is used as the co-surfactant. Stable dispersions can often be formulated over a wide range of oil and water volume fractions. In the regions of the phase diagram containing low concentrations of either oil or water, the structure has frequently been found to be one of oil droplets in water or water droplets in oil². In many cases by simply changing the volume fractions of oil and water, it is possible to effect a gradual transition from an oil-in-water microemulsion to a water-in-oil microemulsion. This raises the question of the structure of the microemulsion in the intermediate region where there are approximately equal volumes of oil and water, and also that of the mechanism of the structural inversion. Microemulsions of such a type are frequently thought of as being constituted by ill-defined oil and water regions arranged with an almost total absence of long-range order³. However, I now report neutron small-angle scattering measurements⁴ on one such microemulsion containing tetradecyl trimethylammonium bromide, butanol, octane and water which shows a well defined diffraction pattern consistent with a cubic arrangement of oil and water domains. This result shows that microemulsions of this type can be considerably more structured than presently thought and can sometimes resemble cubic liquid crystals.

Figure 1a shows the neutron small-angle scattering spectrum measured from a microemulsion containing approximately equal volumes of oil and water and having the following overall composition: tetradecyl trimethylammonium bromide (2.1 g), butanol (1.5 ml), octane (4 ml) and water (4 ml). The microemulsion is optically isotropic and of low viscosity. The spectrum shows a peak similar to that observed in other microemulsions of similar composition^{4,5}. The radius of curvature of the spheroidal domains, as estimated from the scattering curve, is 50 Å. Consistent with its low viscosity, this microemulsion has little or no long-range order and individual Bragg peaks are not observed. A second system was therefore formulated whereby the extent of long-range order in the microemulsion was increased by decreasing the alcohol content. Addition of alcohol to surfactant-water lyotropic liquid crystals tends to destroy the long-range order in these mesophases. As there is a relatively large amount of alcohol (2.5 molecules of butanol for each molecule of surfactant) in the first microemulsion, one may expect the reverse process to occur—reducing the alcohol content could increase the long-range order in the system and lead to the appearance of resolved Bragg peaks in the neutron spectrum. This turns out to be the case. Figure 1b shows the neutron small-angle spectrum from a dispersion containing 30% less alcohol than before (1 ml of butanol instead of 1.5 ml). The microemulsion is now extremely viscous but remains optically isotropic. The broad envelope seen in Fig. 1a has split and at least five diffraction peaks are clearly resolved. The position of the most intense peak remains unchanged and this suggests that, apart from the extent of long-range order, the structure of the microemulsion is unaffected by reducing the alcohol content. The diffraction pattern shown in Fig. 1b indexes onto a cubic lattice. In cubic liquid crystals it is difficult to distinguish between primitive, face-centred, body-centred and diamond-like structures; however, the observed spectrum seems to be in better agreement with a diamond arrangement than with the others.

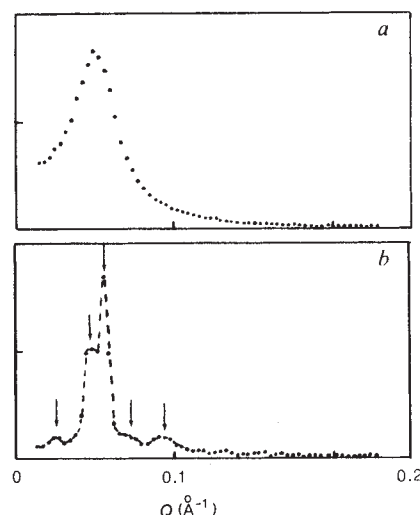


Fig. 1 Neutron small-angle scattering spectra from microemulsions containing equal volumes of oil and water (tetradecyl trimethylammonium bromide, 2.1 g; octane, 4 ml; and water, 4 ml) but having different butanol contents: a, 1.5 ml; b, 1.0 ml. The spectrum shown in b indexes onto a cubic structure. The spectra were measured using the D16 camera¹⁰ at the Institut Laue-Langevin, Grenoble on samples containing D₂O as the only deuterated component.

In the phase diagram, the viscous isotropic phase borders onto a birefringent phase which may have a lamellar structure, while the fluid phase of higher alcohol content meets up at lower surfactant concentrations with microemulsions which show Winsor I–Winsor III–Winsor II phase transitions with added salt⁶. A similar phase behaviour occurs for the SDS/butanol/toluene/water system. The viscous isotropic phase obtained in this system also shows a diffraction pattern similar to that shown in Fig. 1b, and such cubic structures may well be a general feature of this type of microemulsion.

Of the different possible cubic structures, the most likely appears to be the interwoven bicontinuous arrangement proposed by Scriven⁷. However, the possibility that the microemulsion is comprised of an organized dispersion of oil or water or droplets cannot, for the time being, be discounted. The latter type of structure would support the interpretation of Kotlarchyk *et al.*⁵, whose results were obtained using a different type of surfactant.

The observation of cubic diffraction patterns from a microemulsion containing equal volumes of oil and water, shows not only that the oil and water domains are arranged on a cubic lattice, but also that under some circumstances considerable long-range order may exist. Rather than having ill-defined structures, some of these microemulsions show a strong resemblance to the cubic liquid crystalline mesophases^{8,9} that occur in surfactant–water binary mixtures.

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1. Prince, L. M. *Microemulsions. Theory and Practice* (Academic, New York, 1977).
2. Cebula, D. J., Harding, L., Ottewill, R. H. & Pusey, P. N. *Colloid Polym. Sci.* **258**, 973–97x (1980).
3. Talmon, Y. & Prager, S. *J. chem. Phys.* **69**, 2984 (1978).
4. de Geyer, A. & Tabony, J. *Chem. Phys. Lett.* **113**, 83 (1985).
5. Kotlarchyk, M., Chen, S. H., Huang, J. S. & Kim, M. W. *Phys. Rev. Lett.* **53**, 941 (1984).
6. Winsor, P. A. *Solvent Properties of Amphiphilic Compounds* (Butterworths, London, 1954).
7. Scriven, L. E. *Nature* **263**, 123 (1976).
8. Elkwall, P. in *Advances in Liquid Crystals* (ed. Brown, G. H.) Vol. 1, Ch. 1 (Academic, New York, 1971).
9. Tiddy, G. J. T. *Phys. Rep.* **59** (1980).
10. *Neutron Beam Facilities Available for Users* (Institut Laue-Langevin, Grenoble, 1981).

Electroreception and electrolocation in platypus

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Electroreceptors with sensitivity in the microvolt range, which mainly function to detect live prey, are well known in phylogenetically old fishes and some amphibians¹⁻⁴. In African mormyrid and South American gymnotiform fishes this sense has evolved to an active system using an electric organ as a source for impedance measurement of the environment and for communication^{5,6}. Electroreception in higher vertebrates has not previously been reported. Here we establish that the platypus, the Australian nocturnal diving monotreme, can locate and avoid objects on the basis of d.c. fields. High-frequency sensitivity to a.c. could allow the detection of muscle activity of animals, such as crustaceans, which are preyed on by the platypus. Recordings of cortical evoked potentials showed that the bill of the platypus, previously considered to be exclusively mechanoreceptive⁷⁻⁹, is also an electroreceptive organ with behavioural and electrophysiological sensitivity of $\sim 50 \mu\text{V cm}^{-1}$. Several lines of evidence suggest that electroreception has evolved independently in this monotreme.

One male and three female platypuses (*Ornithorhynchus anatinus*) were kept in a round pool filled with conditioned tap water (diameter 3 m, depth 40 cm, resistivity 14 k Ω cm). Hungry

platypuses tended to circle the bottom of the pool, with eyes, ear canals and nares closed, keeping one forefoot in contact with the wall. The bill made 2-3 undulatory sweeps per second parallel to the bottom, which characterized the 'patrol' phase. This became the 'search' phase when the bill made contact with interesting objects, traces of food smell in the water (presumably mediated by the large vomero nasal organ) and weak electric dipole fields, whereas NaCl tablets did not elicit search behaviour. The search phase consisted of erratic movements of the bill over a restricted area of the bottom of the pool and resulted in the platypus homing-in on a freshly killed shrimp or an electric dipole. The sequence was completed by an 'attack' phase in which the object of interest was seized with rapid snaps of the bill.

We used the change from patrol to search phase as a quantitative measure of the ability of the platypus to localize a 1.5-V miniature alkaline battery with a measured dipole field placed on the bottom of the pool at different locations and at systematically varied radial distances from the pool wall. In 88 trials, one animal showed 100% success in the detection of a source 10 cm away with a gradient of 2 mV cm^{-1} (Fig. 1d). The working range extended down to $300 \mu\text{V cm}^{-1}$ at 30 cm, when search bouts were seen only occasionally. Successful detection of the battery was reinforced with a piece of shrimp after every fourth trial in which the battery was attacked. Control experiments offering a choice between a battery, a piece of shrimp tail or a dead battery placed 10 cm apart from each other established a clear preference for the active battery. The possibility that the battery was located chemically by the products of electrolysis near the battery terminals cannot be completely excluded but seems unlikely, especially in view of the experiments described below.

In a second experiment, two 40×40-cm aluminium plates were placed in the pool 3 m apart and connected to the 1.5-V

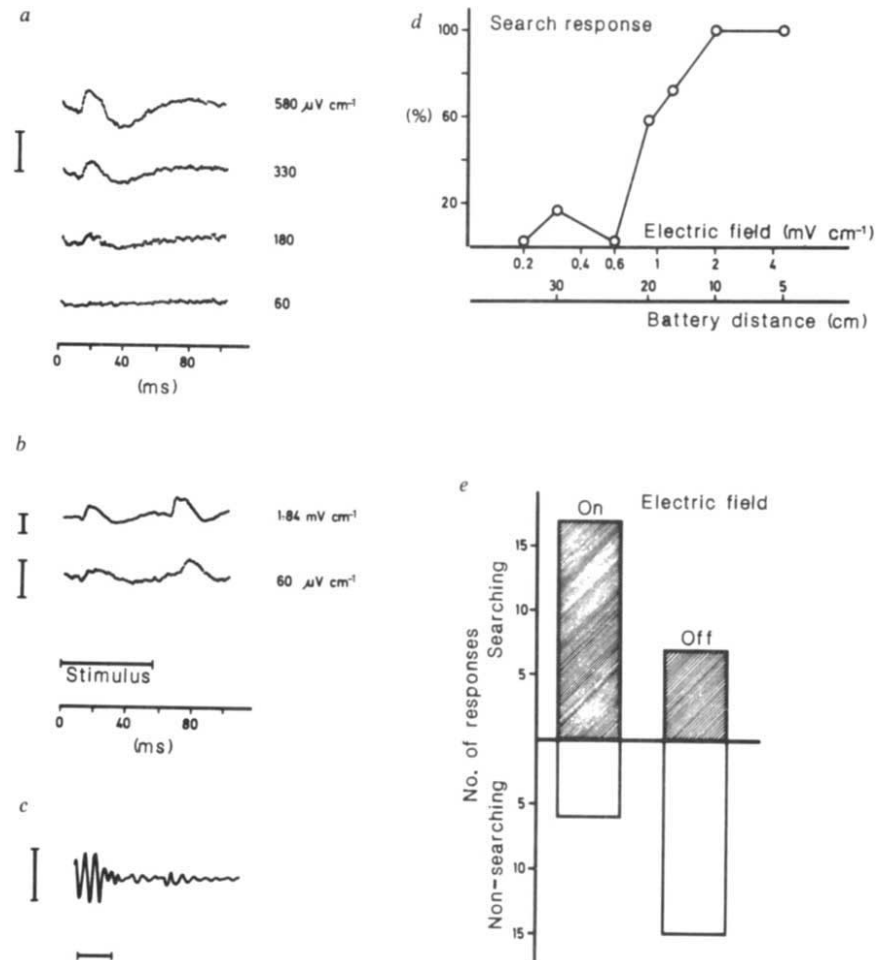


Fig. 1 *a*, Cortical evoked potentials in response to 1-ms rectangular pulse stimulation vertically across the bill of platypus. Series with decreasing voltage. Potentials represent the average response to 1,000 stimuli delivered at 6 s^{-1} . Scale bar, $300 \mu\text{V}$. *b*, Stimulation with pulses of 55 ms duration. Scale bars, $300 \mu\text{V}$. *c*, Compound action potentials recorded differentially with silver wires in the water during tail flicks of *M. australiense* at a distance of 5 cm. The peak power is close to $140 \text{ Hz} = 1/(7 \text{ ms})$, that is, in the range of electrophysiological sensitivity shown in *a* and *b*. Scale bars: vertical, 1 mV; horizontal, 20 ms. *d*, Behavioural detection of the dipole field of a 1.5-v miniature battery as a function of distance. Note that once a search was elicited the battery was invariably found and attacked ($n=88$). *e*, Search in a hollow brick when the platypus passed through a gradient of $330 \mu\text{V cm}^{-1}$ ($P<0.01$, χ^2 -test).

battery through an attenuator. The combined effects of circuit resistance and very large electrodes minimized the current density at the electrode surface, ensuring that electrode potentials and polarization were of negligible proportions. Switching of the field elicited reflex movements of the heads and tails of the animals. Threshold values, when the animals were in approximately the centre of the homogeneous field, were $500 \mu\text{V cm}^{-1}$ for the male and 500, 100 and $50 \mu\text{V cm}^{-1}$ for the females, respectively ($50 \mu\text{V cm}^{-1} \pm 4 \text{ nA cm}^{-2}$). The switches include transients estimated by fast-sweep cathode-ray oscilloscope to be 25% of the plateau voltage.

In a third experiment, one animal learned to avoid a vertical plastic plate that had carbon electrodes attached at its end, carried a d.c. current and was 40 cm wide and as deep as the water. The plate was placed in the pathway during the patrol phase of the underwater search and no food reinforcement given. The animal detoured around the plate at a distance corresponding to a d.c. field gradient of $<200 \mu\text{V cm}^{-1}$, but regularly bumped into the plate when the field was switched off.

In a fourth experiment (Fig. 1e), we made use of the fact that platypuses investigate hollow bricks and sometimes turn them over, particularly when shrimps are hiding inside. The same behaviour was elicited by switching on a field between two carbon electrodes placed behind a brick ($30 \times 15 \times 10 \text{ cm}$) near the wall of the pool. The search occurred in 74% of the trials, when the transients had a gradient of $330 \mu\text{V cm}^{-1}$, measured in front of the brick, compared with 32% when there was no field.

As well as establishing thresholds for electroreception, these four experiments suggest that the platypus can make use of d.c. and a.c. fields in different behavioural contexts, such as object location and avoidance. Electric fields generated by live animals could be used to find food, as has previously been demonstrated for electrosensitive fishes and amphibians^{3,4,10,11}. We have found that the tail flicks of a freshwater shrimp (*Macrobrachium australiense*), which formed the main diet of the platypus used in our study, produce compound action potentials that vary between 0.2 and 1 mV cm^{-1} at a distance of $\sim 5 \text{ cm}$ (Fig. 1c). Platypuses feed exclusively on live prey, including several species of decapod crustaceans, frogs and small fishes¹²⁻¹⁴. As the tail flick of the shrimp moves the animal only a few centimetres, the myogenic electric fields probably provide a guide to its position when it is disturbed by a platypus probing blindly in the mud or among stones.

Finally, the electrosensitivity of the bill was monitored using cortical evoked potentials (e.ps). A previous study⁷ of somatosensory, visual and auditory e.ps provides a comprehensive frame of reference. Recordings were made from the exposed left posterior hemisphere of two animals under anaesthesia (Hypnodil 25 mg per kg body weight plus Ketanest 5 mg per kg per h). The dorsal and ventral surfaces of the upper bill were covered with a thick cushion of wet cotton, keeping the nostrils free for breathing. The side of the bill contralateral to the exposed cortex was stimulated dorsoventrally across the cotton layers with a pair of Ag/AgCl electrodes. The total resistance of the circuit was of the order of 100 k Ω . The maximal voltage gradient for transient-free rectangular pulses was calibrated with a pair of recording electrodes orientated vertically between the stimulating electrodes, using the same pulse-generating system. Applying a 1-ms pulse stimulation across the proximal third of the bill produced a focus of activity on the posterior-lateral hemisphere with a threshold $<180 \mu\text{V cm}^{-1}$ and a maximum of $300 \mu\text{V}$ with a voltage gradient of $580 \mu\text{V cm}^{-1}$ (Fig. 1a). With a 55-ms pulse, on- and off-responses could be distinguished (Fig. 1b), with a threshold of $80 \mu\text{V cm}^{-1}$ in one animal and below $60 \mu\text{V cm}^{-1}$ in the other. To relate the electroreceptive cortical area to the sensory maps previously described⁷, auditory e.ps were recorded in one platypus in response to acoustic clicks (0.5 ms duration, 8 s^{-1} , 80 dB sound pressure level). Short-latency auditory e.ps were recorded in an area of the dorso-caudal cortex corresponding to that of ref. 7. The largest

electroreceptive e.p. was recorded 5 mm ventral to the largest auditory e.p. Rostrally, the electroreceptive e.p. decreased rapidly in amplitude when recording in what seemed to be the area receiving somatosensory inputs from the bill⁷.

Although we identified the bill as the electroreceptive organ, the question of which type of receptor might be used to detect electrical currents remains open. However, recent electron microscope studies of receptor specializations in the bill of the platypus¹⁵ have revealed gland duct receptors. These receptors are unique to platypuses but occur in a simpler form in the echidna. The receptors consist of small protoplasmatic protrusions from myelinated axons of trigeminal neurones, which form filamentous processes extending into the ducts of serous or mucous skin glands. This arrangement is ideal for electroreception in a semi-aquatic animal. Because the epidermis of the bill dries out when the platypus leaves the water, electroreceptors associated with a gland help to preserve conductivity and to prevent damage by desiccation.

All electroreceptors found in fishes and amphibians that do not have electric organs are of the ampullary receptor type^{1,2,13,16} and probably derive from the acoustic-lateralis system^{17,18}. The postulated electroreceptors in platypus belong to the trigeminal system, with a cortical representation next to the trigeminal map of the bill mechanoreceptors. Their sensitivity to 1-ms pulses contrasts with the d.c. or low-frequency responsiveness of ampullary receptors, which cannot detect muscle action potentials. Together with the unique morphology of the receptors, these features suggest that electroreception has evolved independently in this group of lower mammals.

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1. Bullock, T. H. *A. Rev. Neurosci.* **5**, 121-170 (1982).
2. Fritsch, B. & Wahnschaffe, U. *Cell Tissue Res.* **229**, 483-503 (1983).
3. Kalmijn, A. J. in *Handbook of Sensory Physiology* Vol. III/3, 147-200 (Springer, Berlin, 1974).
4. Scheich, H. in *Biophysics*, 764-776 (Springer, Berlin, 1983).
5. Heiligenberg, W. Principles of Electrolocation and Jamming Avoidance in Electric Fish (Springer, Berlin, 1977).
6. Scheich, H. & Bullock, T. H. in *Handbook of Sensory Physiology* Vol. III/3 201-256 (Springer, Berlin, 1974).
7. Bohringer, R. C. & Rowe, M. J. *J. comp. Neurol.* **174**, 1-14 (1977).
8. Poulton, E. B. *J. Physiol., Lond.* **5**, 15-16 (1885).
9. Wilson, J. T. & Martin, C. J. *Linn. Soc. NSW, Macleay mem. Vol.*, 190-200 (1893).
10. Digkgraaf, S. & Kalmijn, A. J. *Z. vergl. Physiol.* **47**, 438-456 (1963).
11. Himstedt, W., Kopp, J. & Schmidt, W. *Naturwissenschaften* **69**, 552 (1982).
12. Crowther, A. B. *Pap. Proc. R. Soc. Tasman.* **96**-99 (1879).
13. Faragher, R. A., Grant, T. R. & Carrick, F. N. *Aust. J. Ecol.* **4**, 171-179 (1979).
14. Grant, T. R., Williams, R. & Carrick, F. N. *Aust. Zool.* **19**, 117-124 (1977).
15. Andres, K. H. & von Düring, M. in *Sensory Receptor Mechanisms* 81-89 (World Science, Singapore, 1984).
16. Münz, H., Claas, B. & Fritsch, B. *J. comp. Physiol.* **A154**, 33-44 (1983).
17. Bennett, M. V. L. in *Fish Physiology*, 492-600 (Academic, London, 1971).
18. Szabo, T. in *Handbook of Sensory Physiology* Vol. III/3, 13-58 (Springer, Berlin, 1974).

Acetylcholine induces burst firing in thalamic reticular neurones by activating a potassium conductance

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Recent studies have emphasized the role of acetylcholine (ACh) as an excitatory modulator of neuronal activity in mammalian cortex¹⁻⁵ and hippocampus⁶⁻⁹. Much less is known about the mechanism of direct cholinergic inhibition in the central nervous system or its role in regulating neuronal activities. Here we report that application of ACh to thalamic nucleus reticularis (nRt) neurones, which are known to receive a cholinergic input from the ascending reticular system of the brain stem^{10,11}, causes a hyper-

polarization due to a relatively small (1–4 nS) increase in membrane conductance to K^+ . This cholinergic action appears to be mediated by the M_2 subclass of muscarinic receptors and acts in conjunction with the intrinsic membrane properties of nucleus reticularis neurones to inhibit single spike activity while promoting the occurrence of burst discharges. Thus, cholinergic inhibitory mechanisms may be important in controlling the firing pattern of this important group of thalamic neurones.

Application of ACh to thalamic nucleus reticularis neurones recorded intracellularly in thalamic slices maintained *in vitro*^{12,13} resulted in a hyperpolarization with a minimum onset latency of approximately 80 ms and a duration of 5–20 s (Fig. 1a). The amplitude of the current induced by ACh and changes in the apparent input conductance (G_i) were estimated by manually clamping the membrane potential (V_m) at resting level while applying hyperpolarizing conductance test pulses (Fig. 1b). Using this approach we found that ACh caused an increase in G_i of 1–4 nS and an estimated outward current of 25–75 pA. This ACh-induced change in G_i was dose-dependent, but was less than 6 nS even with applications of large amounts of ACh (500–1,000 times the typical threshold dose).

To test whether the hyperpolarizing response to ACh was due to a direct action on the cell studied or was mediated through the release of another neurotransmitter^{4–7}, we blocked synaptic transmission by bathing the slices in solution containing low Ca^{2+} (0.5 mM) and Mn^{2+} (2 mM), or by local application of tetrodotoxin (10 μ M). Neither of these manipulations blocked the ACh-induced inhibition ($n=8$), although the response to an orthodromic stimulus was abolished completely. Therefore, this effect of ACh appears to be due to interaction with receptors located directly on the cell studied.

The results of previous extracellular studies indicate that the actions of ACh on nRt neurones are mediated by muscarinic receptors¹⁴. In support of this, we found that local applications (10 μ M; $n=3$) of the specific muscarinic antagonist scopolamine completely blocked the ACh-induced hyperpolarization and applications of the specific muscarinic agonist acetyl- β -methacholine (5 mM; $n=4$) mimicked the actions of ACh (not shown). Furthermore, these responses appear to be mediated by the M_2 subclass of muscarinic receptors as we found that bath applications of the specific M_1 antagonist pirenzepine^{15,16} in doses more than sufficient to block M_1 -mediated responses in the cerebral cortex (10 and 100 μ M; $n=7$)⁴ failed to block the ACh response of nRt neurones. This finding agrees with recent autoradiographic data which show that the nRt contains muscarinic receptors of the M_2 subtype^{16,17}.

The ACh-induced hyperpolarization could be due to an increase in membrane conductance to K^+ or Cl^- or a decrease in a voltage-dependent depolarizing current (that is, Na^+ or Ca^{2+}). To distinguish between these possibilities, the effect of changes in V_m on the ACh-induced hyperpolarization was assessed. Hyperpolarization of V_m produced by intracellular current injection decreased the amplitude of the hyperpolarizing response to ACh and revealed that the response had a mean reversal potential of -89 mV (± 5.8 mV, s.d.; $n=8$; Fig. 2) in 5.0 mM K. This effect of V_m on the ACh response indicates an increase in membrane conductance to either K^+ or Cl^- . If the change in membrane conductance induced by ACh was primarily to K^+ , then changing the extracellular K^+ concentration ($[K^+]_o$) should effect the equilibrium potential of the ACh-induced response as predicted by the Nernst equation (61 mV shift in E_{ACh} per 10-fold change in $[K^+]_o$). The average reversal potential of the ACh-induced hyperpolarization was -104 mV (± 4.8 ; $n=4$) in 2.5 mM K^+ and -75 mV (± 2.9 ; $n=4$) in 7.5 mM K^+ . By extrapolation, E_{ACh} was calculated to change by 58 mV for each 10-fold change in $[K^+]_o$ (Fig. 2b). In contrast, the average reversal potential of responses to γ -aminobutyric acid (GABA), another inhibitory neurotransmitter which typically increases membrane Cl^- conductance, ranged from -62 to -67 mV and varied by only 11 mV for each 10-fold change in

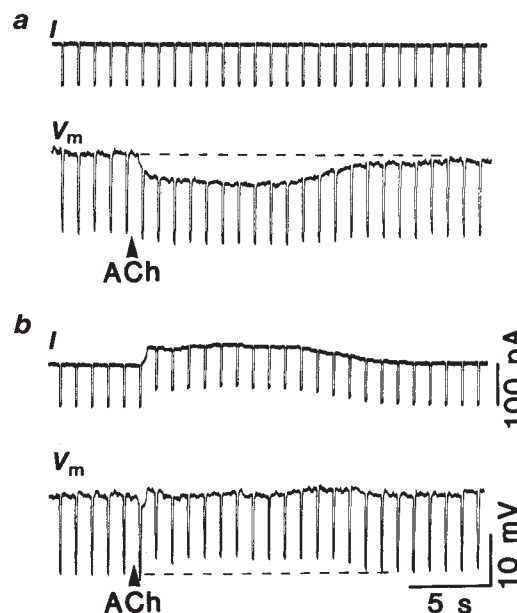


Fig. 1 Effect of ACh on membrane potential and apparent input conductance of nRt neurones. *a*, During constant current recordings, application of ACh resulted in a hyperpolarization. *b*, Manual compensation for the slow hyperpolarization using intracellular injection of depolarizing current during a second application of ACh to the neurone of *a*. Approximately 50 pA of compensating current was required and the response was associated with an apparent increase in input conductance of ~ 2 nS.

Methods. Intracellular recordings from nRt neurones were obtained using standard techniques^{5,6}. Coronal thalamic slices, 500 μ m thick, containing the anterior portions of the Rt nucleus were prepared on a Vibratome from tissue obtained from anaesthetized male or female guinea pigs. Slices were maintained in an interface type chamber at $36 \pm 1^\circ$ C. Slice bathing solution contained (in mM): NaCl, 124; KCl, 5; $MgSO_4$, 2; $NaHCO_3$, 26; NaH_2PO_4 , 1.25; $CaCl_2$, 2; glucose, 10 and was saturated with 95% O_2 , 5% CO_2 to a final pH of 7.4. ACh (5 mM) and GABA (1 mM) were applied from a broken micropipette (2 μ m tip diameter) by pressure pulses (typically 20 ms) in volumes of 1–10 pl, within 75 μ m of the recorded neurone. Microelectrodes were filled with either 4 M KAc or 3 M KCl and bevelled to final resistances of 100–200 M Ω . Apparent input conductance was monitored with intracellular current pulses (typically 0.1 nA, 120 ms) delivered at a rate of 1 Hz. Extracellular single unit recordings were obtained with coated tungsten electrodes (Frederick-Haer). When testing the effects of ACh on neuronal responses to synaptic stimulation or applications of glutamate, these excitatory inputs were adjusted to yield only one or two spikes. Synaptic stimuli were delivered through a bipolar concentric stimulating electrode located in the white matter just lateral to the nRt. When Mn^{2+} was added to the bathing solution, sulphate and phosphate were omitted to prevent precipitation. When the concentration of K^+ was varied, the concentration of Na^+ was changed to maintain the same osmolarity. The nRt was identified in the guinea pig by immunohistochemical staining for glutamic acid decarboxylase (GAD)¹⁹. Neurones within this immunoreactive nucleus were readily distinguished from other thalamic neurones by their position within the slice and their distinctive electrophysiological properties which included action potentials of unusually brief duration (0.4–0.8 ms at base) and the ability to fire at high frequencies (>500 Hz).

$[K^+]_o$ (Fig. 2b), probably due to secondary shifts in Cl^- distribution¹⁸. These results suggest that the ACh-induced hyperpolarization is due to an increase in membrane K^+ conductance. To test this hypothesis further, neurones were impaled with KCl containing electrodes and Cl^- iontophoresed intracellularly by passing negative current (0.25–0.5 nA) through the electrode. Application of GABA to neurones under such circumstances resulted in large depolarizations that caused the generation of action potentials and had reversal potentials which were always well above firing threshold (that is positive to -50 mV; $n=7$),

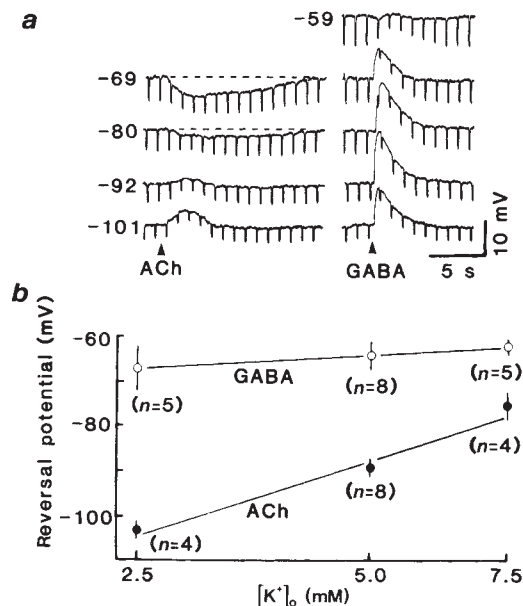


Fig. 2 Reversal potentials for responses to ACh and GABA and the effect of extracellular K^+ concentration on these. *a*, Application of ACh to this neurone in normal solution (5 mM K) caused a hyperpolarization which had a reversal potential of ~ -90 mV (*a*, left). The duration of the ACh response appeared to shorten during large current-induced hyperpolarizations (-92 and -101 mV). The ACh responses of -92 and -101 mV appear to be slightly biphasic, although this effect was small and inconsistent and therefore could not be investigated thoroughly. In contrast, applications of GABA to this same neurone caused a depolarization which had a reversal potential of ~ -59 mV (*a*, right). The responses to ACh and GABA in this cell were obtained after blockade of Na^+ -mediated action potentials with the local application of tetrodotoxin ($10 \mu M$). *b*, Group data illustrating the effect of varying the concentration of K^+ in the bathing medium on the reversal potentials of ACh- and GABA-induced responses. The reversal potential for ACh-induced responses changed by 58 mV for each 10-fold increase in $[K^+]_o$, while that for responses induced by GABA was shifted by only 11 mV, indicating that the ACh, but not the GABA, response was probably due primarily to an increase in conductance to K^+ . The plotted data are mean $\pm$ s.e.m.

indicating that a positive shift in E_{Cl} of at least 20 mV had occurred. In these conditions applications of ACh still caused hyperpolarizations when membrane potentials were manipulated between -65 and ~ -90 mV ($n = 7$), and the average E_{ACh} was -89 mV (± 2.5 ; $n = 3$). These results confirm that the membrane conductance activated by ACh is predominantly for K^+ ions.

Although the above actions of ACh and GABA are, strictly speaking, both inhibitory in nature, the intrinsic properties of nRt neurones are such that the net effect on neuronal activity is quite different for these two agents. When compared with neurones of other thalamic nuclei (see also refs 12, 13), neurones of nRt display unique electrophysiological properties including high input resistance (typically 75–250 M Ω) and an apparent high specific membrane resistivity (as evidenced by relatively long membrane time constants of 20–50 ms). In addition, we found that, like other thalamic neurones^{12,13}, nRt cells can generate a low-threshold, tetrodotoxin-resistant, slow spike which underlies burst discharges. This potential is inactivated near or above firing threshold, but can be de-inactivated by hyperpolarization of V_m to 5–20 mV below firing threshold^{12,13}. As a consequence of these intrinsic properties of nRt neurones, the relatively small K^+ current induced by ACh was effective in causing a substantial shift in V_m towards E_K and consequently deactivating the low-threshold Ca^{2+} spike and associated burst discharges. Thus, application of ACh to single nRt neurones recorded extracellularly resulted in inhibition of spontaneous

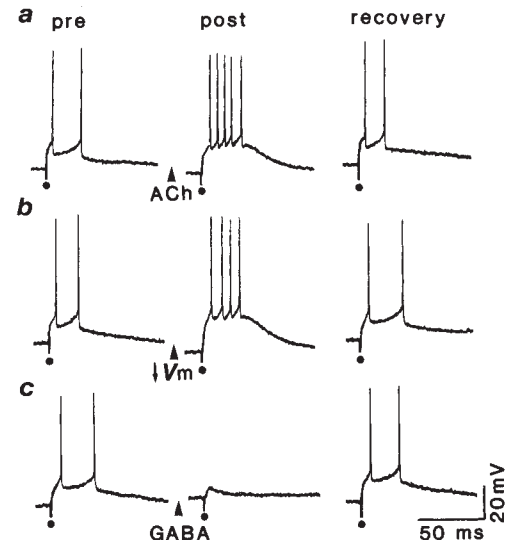


Fig. 3 Effect of ACh, GABA and change in membrane potential on the response of a nucleus reticularis neurone to orthodromic synaptic inputs. *a*, Application of ACh at upward-pointing arrow causes a membrane hyperpolarization, presumably through an increase in K^+ conductance, and changes the response of the neurone to the synaptic stimulus (dot) from two action potentials (pre) to a burst of five (post). After ~ 15 s, the response of the neurone returns to normal (recovery). *b*, Hyperpolarizing the membrane potential through the intracellular injection of current alone has a similar effect on the response of this neurone to the synaptic stimulus. *c*, In contrast, application of GABA, which presumably causes an increase in Cl^- conductance, depolarizes the membrane potential and inhibits all spike activity. Synaptic stimuli were of constant amplitude and delivered at 0.2 Hz. All records from the same nRt neurone.

activity and, in some cells, an increased frequency of spike burst discharge, as has been reported previously *in vivo*¹⁴. Furthermore, if ACh was applied during orthodromic stimulation (Fig. 3*a*), during pressure pulse applications of glutamate (0.5 mM), or during intracellular depolarizing current pulses, the response of the neurones to these excitatory inputs would change from single spike activity to a burst response of 3–8 spikes. A similar effect occurred when V_m was hyperpolarized with the intracellular injection of current alone (Fig. 3*b*). In contrast, applications of GABA caused inhibition of all spike activity by evoking an increase in membrane conductance to Cl^- , which shifted V_m away from the potentials at which the burst mechanism is active (Fig. 3*c*).

Because of their widespread projection to thalamic relay and intralaminar nuclei and the presence of diverse inputs from corticothalamic and thalamocortical axons²⁰, the GABAergic neurones of the nRt¹⁹ are ideally situated to influence excitability of both thalamus and cortex²⁰. Our results, and others¹⁴, indicate that the portion of the ascending brainstem reticular system which is cholinergic in nature may have a major role in these interactions by controlling the relative dominance of single spike versus burst firing patterns of activity of neurones in the nucleus reticularis of the thalamus.

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Note added in proof: We have recently found that applications of ACh to neurones of the lateral and medial geniculate yield a hyperpolarization virtually identical to that reported here, indicating that ACh induced increases in K conductance may be a general mechanism for cholinergic inhibition in the thalamus.

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1. Constanti, A. & Galvan, M. *Neurosci. Lett.* **39**, 65–70 (1983).
2. ffrench-Mullen, J. M. H., Nori, H., Nakanishi, H., Slater, N. T. & Carpenter, D. O. *Cell molec. Neurobiol.* **3**, 163–181 (1983).
3. Krnjevic, K., Pumain, R. & Renaud, L. *J. Physiol. Lond.* **215**, 447–465 (1971).
4. McCormick, D. A. & Prince, D. A. *Proc. natn. Acad. Sci. U.S.A.* **82**, 6344–6348 (1985).
5. McCormick, D. A. & Prince, D. A. *J. Physiol. Lond.* (in the press).
6. Bernardo, L. S. & Prince, D. A. *Brain Res.* **249**, 315–333 (1982).
7. Bernardo, L. S. & Prince, D. A. *Brain Res.* **249**, 334–344 (1982).
8. Cole, A. E. & Nicoll, R. A. *J. Physiol. Lond.* **352**, 173–188 (1984).
9. Halliwell, J. V. & Adams, P. R. *Brain Res.* **250**, 71–92 (1982).
10. Fibiger, H. C. *Brain Res. Rev.* **4**, 327–388 (1982).
11. Mesulam, M.-M., Mufson, E. J., Wainer, B. H. & Levey, A. I. *Neuroscience* **10**, 1185–1201 (1983).
12. Jahnsen, H. & Llinas, R. *J. Physiol. Lond.* **349**, 205–226 (1984).
13. Jahnsen, H. & Llinas, R. *J. Physiol. Lond.* **349**, 227–247 (1984).
14. Ben-Ari, Y., Dingledine, R., Kanazawa, I. & Kelly, J. S. *J. Physiol. Lond.* **261**, 647–671 (1976).
15. Hammer, R., Birdsall, N. J. M., Burgen, A. S. V. & Hulme, E. C. *Nature* **283**, 90–92 (1980).
16. Birdsall, N. J. M., Hulme, E. C. & Stockton, J. M. *Trends pharmac. Sci. Suppl.*, 4–8 (1983).
17. Wamsley, J. K., Zarbin, M. A. & Kuhar, M. J. *Brain Res. Bull.* **12**, 233–243 (1984).
18. Deisz, R. A. & Lux, H. D. *J. Physiol. Lond.* **326**, 123–138 (1982).
19. Houser, C. R., Vaughn, J. E., Barber, R. P. & Roberts, E. *Brain Res.* **200**, 341–354 (1980).
20. Steriade, M. & Deschenes, M. *Brain Res. Rev.* **8**, 1–63 (1984).

Acetylcholine hyperpolarizes central neurones by acting on an M_2 muscarinic receptor

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Acetylcholine (ACh) is considered to act as a neurotransmitter in the mammalian brain by binding to membrane receptors and bringing about a change in neurone excitability. In the case of muscarinic receptors, cell excitability is usually increased¹; this effect results from a closure of membrane potassium channels in cortical cells^{2,3}. However, some central neurones are inhibited by ACh⁴, and we hypothesized that these two opposite effects of ACh resulted from interactions with different subtypes of muscarinic receptor. We made intracellular recordings from neurones in the rat nucleus parabrachialis, a group of neurones in the upper pons some of which themselves synthesize ACh^{4,5}. ACh and muscarine caused a membrane hyperpolarization which resulted from an increase in the membrane conductance to potassium ions. The muscarinic receptor subtype was characterized by determining the dissociation equilibrium constant (K_D) for pirenzepine during the intracellular recording; the value of ~ 600 nM indicates a receptor in the M_2 class. This muscarinic receptor is quite different from that which brings about a decrease in potassium conductance in other neurones, which has a pirenzepine K_D of ~ 10 nM (M_1 receptors)^{6–8}. It is possible that antagonists selective for this kind of M_2 receptor would be useful in the management of conditions, such as Alzheimer's disease, which are associated with a reduced effectiveness of cholinergic neurones.

Intracellular recordings were made from neurones of the nucleus parabrachialis contained within a slice cut from the pons of a rat and superfused *in vitro* with a physiological saline solution (see Fig. 1 legend and ref. 9). The parabrachial neurones had resting membrane potentials of -65.6 ± 1.1 mV (mean $\pm$ s.e.m., $n = 17$) and apparent input resistances of 200–400 M Ω . Many neurones exhibited spontaneous depolarizations which were apparently synaptic potentials (they were blocked in calcium-free superfusion solutions) and these occasionally gave rise to action potentials.

ACh hyperpolarized the membrane when it was added to the superfusion solution, or when it was ejected from a pipette onto the surface of the tissue slice. The amplitude of the hyperpolarization depended on the concentration of ACh applied and readily attained 20–25 mV (Fig. 1a). The effective concentrations were 100 nM to 30 μ M in the presence of neostigmine (1 μ M), and 10–300 μ M in the absence of neostigmine. This effect of neostigmine indicates that a substantial proportion of the added ACh

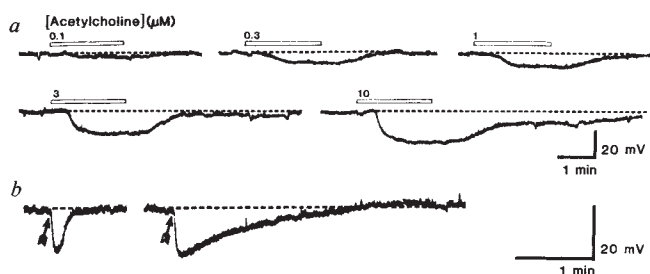


Fig. 1 Acetylcholine (ACh) hyperpolarizes neurones of rat nucleus parabrachialis. Records show membrane potential (resting level -60 mV). *a*, The bar above each trace indicates the period during which the superfusion solution contained ACh (concentration indicated); the solution also contained neostigmine (1 μ M) throughout this experiment. Approximately 5 min elapsed between each of the traces shown. The neurones did not fire action potentials spontaneously during the hyperpolarization evoked by ACh, and it was difficult to evoke action potentials by passing depolarizing current pulses, presumably because of the increased membrane conductance (see Fig. 2). *b*, The arrows indicate the time of application of a pressure pulse (70 kPa, 10 ms) to a pipette that contained ACh chloride (1 mM), the tip of which (diameter 10 μ m) was positioned beneath the surface of the superfusion solution. Left trace, control. Right trace, the same ACh application in the presence of neostigmine (1 μ M).

Methods. Slices of rat pons containing the nucleus parabrachialis were obtained in a manner similar to that described for the locus coeruleus⁹. Male Sprague-Dawley rats were lightly anaesthetized with ether and killed by a blow to the chest; the brain was quickly removed and placed in physiological saline solution at -4 $^{\circ}$ C. A slice of pons containing the nucleus parabrachialis close to its maximum lateral extent was cut with a Vibratome; the slice also usually contained the rostral pole of the locus coeruleus. The dorsal and ventral divisions of the nucleus could be seen on either side of the superior cerebellar peduncle. The slice was completely submerged in a chamber (capacity ~ 500 μ l) through which flowed a solution, heated to 37 $^{\circ}$ C, of the following composition (mM): NaCl 126, KCl 2.5, NaH_2PO_4 1.2, MgCl_2 1.3, CaCl_2 2.5, NaHCO_3 25, glucose 11, saturated with 95% O_2 /5% CO_2 . Intracellular recordings of membrane potential were made with electrodes containing potassium chloride or potassium acetate. In some experiments, membrane currents were measured directly with a single electrode amplifier which switched from voltage measuring to current-passing mode at a frequency of 3–6 kHz (Axoclamp 11).

is destroyed by cholinesterase in the brain slice before reaching the impaled neurone (Fig. 1b). Muscarine (1–100 μ M) also hyperpolarized parabrachial neurones, the effect being observed in 21 of 32 cells. The muscarinic hyperpolarization persisted during superfusion with a solution which contained decreased calcium (0.6 mM) and increased magnesium (20 mM) concentrations. This implies that the effect of the ACh was not due to the liberation of other transmitters, because such solutions blocked the spontaneous synaptic potentials.

The muscarinic hyperpolarization resulted from an increase in the conductance of the cell membrane to potassium ions. In the first place, ACh caused a fall in the neurone input resistance measured by passing small hyperpolarizing currents (Fig. 2). Second, the hyperpolarization was associated with an outward current measured under voltage clamp. Both the muscarinic hyperpolarization and the current amplitude were linearly dependent on the membrane potential, having zero amplitude at a potential which changed with the logarithm of the potassium concentration in the superfusion solution ($[\text{K}^+]_o$) (Fig. 2). In control solutions ($[\text{K}^+]_o = 2.5$ mM), the reversal potential was -104.4 ± 1.1 mV ($n = 8$) (Fig. 2). Third, muscarinic hyperpolarizations were reproducible for several hours whether the recording electrode contained potassium chloride or potassium acetate. Fourth, the hyperpolarization was blocked by barium (4 mM). Muscarinic hyperpolarizations with similar characteristics have been described previously in peripheral autonomic neurones^{10–12}, but not in the central nervous system.

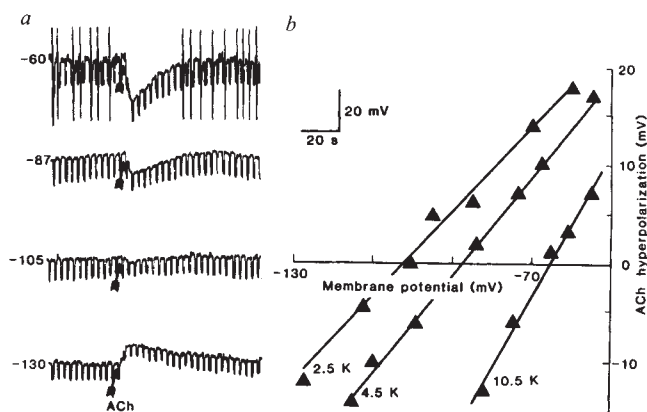


Fig. 2 Acetylcholine increases potassium conductance. *a*, Records of membrane potential; downward deflections are electrotonic potentials evoked by passing current pulses which were constant throughout. Action potentials and their afterhyperpolarizations are also seen in the top trace, but the full amplitude of the action potentials is not reproduced by the pen recorder. The arrows indicate the times of application of ACh. The ACh was ejected from a pipette containing ACh chloride (see Fig. 1 legend). The ACh hyperpolarization became smaller when the membrane was hyperpolarized and reversed polarity at about -105 mV. *b*, Complete results from the same neurone. The voltage dependence of the ACh response was determined first in control (2.5 mM) and then in solutions containing higher potassium concentrations as indicated (in mM).

The hyperpolarizations were completely blocked by atropine and hyoscyne (3 – 100 nM, $n = 4$) but were unaffected by nicotine (50 μ M) or hexamethonium (400 μ M). Neurones located in the nearby locus coeruleus were never hyperpolarized by ACh or muscarinic agonists (see ref. 13); furthermore, noradrenaline and enkephalin, which hyperpolarize locus coeruleus neurones^{14,15}, had no effect on cells of the nucleus parabrachialis.

Pirenzepine is a muscarinic receptor antagonist that has different affinities for the two different subtypes of muscarinic receptors^{16,17}. Pirenzepine did not affect the muscarinic hyperpolarizations in concentrations less than 300 nM ($n = 5$), but higher concentrations caused a parallel shift in the ACh dose-response curve. In three experiments, sufficient agonist and antagonist concentrations were applied for the results to be analysed by the Gaddum-Schild method¹⁸. This indicated that pirenzepine competitively blocked the effect of muscarine, with K_D values of 500 , 590 and 725 nM (Fig. 3). The relatively low affinity of pirenzepine for this receptor indicates that it belongs to the M_2 category^{14,15}. Estimates of the dissociation equilibrium constant of atropine were not made, because previous studies have shown that atropine has the same K_D for both the M_1 and M_2 receptors¹⁹. Our own experiments on single neurones support this. Cells of the rat locus coeruleus (part of which is contained within the same brain slice as the nucleus parabrachialis) are depolarized by muscarinic agonists; the receptors underlying this effect (M_2) have a K_D for atropine of ~ 680 pM and for pirenzepine of 230 nM (ref. 13). On the other hand, neurones in the guinea pig enteric nervous system are depolarized by muscarinic agonists through a different ionic mechanism²⁰. On these cells the muscarinic receptor is the M_1 subtype, having a K_D for pirenzepine of ~ 3 nM, but the K_D for atropine is 1 nM, a value close to that found in the locus coeruleus²¹. In the present experiments we found that a concentration of atropine close to this K_D value (3 nM) prevented or reduced the muscarinic hyperpolarization, suggesting that atropine is not likely to be useful in further characterization of the receptor subtype involved.

ACh acts on muscarinic receptors in excitable tissues to bring about a variety of membrane conductance changes, some of which can now be ascribed to particular receptor subtypes. The

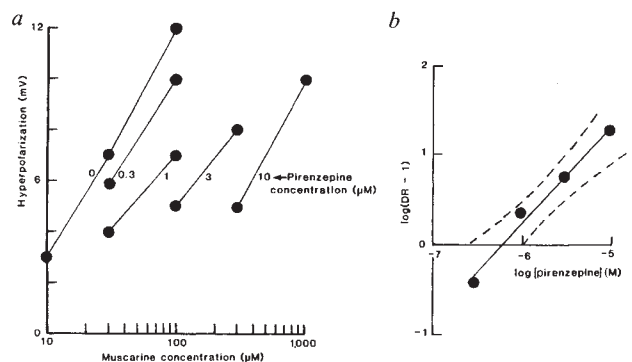


Fig. 3 The muscarinic receptor on parabrachial neurones is of an M_2 subtype. *a*, Hyperpolarizations were evoked by superfusion of various concentrations of muscarine, which was first applied alone and then in the presence of four different concentrations of pirenzepine. All records were obtained during recording from one neurone. *b*, Schild plot for the antagonism of muscarine by pirenzepine. The slope of the line (1.08) was not significantly different from 1 . The pirenzepine K_D was 590 nM. Broken lines are the 95% confidence limits of the line. Two other experiments yielded similar results, with confidence limits of the estimates of the K_D of 200 – $1,100$ nM.

decrease in potassium conductance^{2,3,6,8,20} results from an action on M_1 receptors, which bind pirenzepine with a K_D of ~ 10 nM (refs 6, 17). The increase in potassium conductance reported here results from activation of an M_2 receptor having a pirenzepine K_D of 600 nM; the same value for the pirenzepine K_D has been reported for antagonism of muscarinic slowing of the guinea pig heart²², which probably also results from an increase in potassium conductance of the atrial cells²³. A third effect of muscarinic agonists is a reduction in a voltage-dependent calcium conductance^{24–26}. There are no estimates of a pirenzepine K_D for block of this muscarinic action. However, the negative inotropic effect on the heart (which may correlate with a reduced calcium conductance) appears to involve an M_2 receptor having a pirenzepine K_D of 186 nM, a value which may be different from that obtained when cardiac slowing is measured²². This suggests that the M_2 receptors associated with potassium conductance increase and reduction in calcium conductance may be distinguishable. A fourth type of effect is an increase in membrane conductance, predominantly to sodium ions, which brings about a depolarization of some gland cells²⁷, neurones^{13,28} and smooth muscle cells²⁹. An M_2 receptor is also implicated in one of these effects¹³. It is likely that the further development of antagonists will allow the discrimination among the ' M_2 ' receptors responsible for these distinct effects on ion conductances.

There is considerable evidence for presynaptic location of M_2 receptors on peripheral cholinergic and adrenergic fibres^{6,30,31} and binding sites believed to identify these receptors have been assigned a presynaptic location in the central nervous system^{32,33}. Lesions that result in loss of neurones containing choline acetyltransferase (CAT) also lead to loss of cortical M_2 binding sites, implying that the latter may be 'autoreceptors'³³. It is of interest, therefore, that the neurones hyperpolarized in the present study were in a part of the brain (nucleus parabrachialis) which contains CAT-immunoreactive cells⁴, although it should be stressed that the individual cells from which the recordings were made have not been shown to contain CAT. The potassium conductance increase in the soma will result in strong inhibition of firing, and if a similar effect were to occur close to axon terminal release sites it would reduce the amount of transmitter released. A blockade of these actions of endogenous ACh by selective M_2 receptor antagonists might be useful in the treatment of those disorders, such as Alzheimer's disease, that involve a loss of cholinergic neurones bearing this muscarinic receptor subtype³³.

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- Krnjevic, K. *Physiol. Rev.* **54**, 418–540 (1974).
- Krnjevic, K., Pumain, R. & Renaud, L. *J. Physiol., Lond.* **215**, 247–268 (1971).
- Halliwel, J. & Adams, P. R. *Brain Res.* **250**, 71–92 (1982).
- Armstrong, D. M., Saper, C. B., Levey, A. I., Wainer, B. H. & Terry, R. D. *J. comp. Neurol.* **216**, 53–68 (1983).
- McGeer, P. L., McGeer, E. G. & Peng, J. H. *Life Sci.* **34**, 2319–2338 (1984).
- North, R. A. & Surprenant, A. *J. Physiol., Lond.* **368**, 435–452 (1985).
- Brown, D. A., Forward, A. & Marsh, S. *Br. J. Pharmac.* **71**, 362–364 (1980).
- Brown, D. A. & Constanti, A. *Br. J. Pharmac.* **70**, 593–608 (1980).
- Williams, J. T., North, R. A., Shefner, S. A., Nishi, S. & Egan, T. M. *Neuroscience* **13**, 137–156 (1984).
- Hartzell, H. C., Kuffer, S. W., Stickgold, R. & Yoshikami, D. *J. Physiol., Lond.* **271**, 817–846 (1977).
- Dodd, J. & Horn, J. P. *J. Physiol., Lond.* **334**, 271–291 (1983).
- Cole, A. E. & Shinnick-Gallagher, P. *Nature* **307**, 270–271 (1984).
- Egan, T. M. & North, R. A. *Br. J. Pharmac.* **85**, 733–735 (1985).
- Williams, J. T., Henderson, G. & North, R. A. *Neuroscience* **14**, 95–101 (1985).
- Williams, J. T., Egan, T. M. & North, R. A. *Nature* **299**, 74–76 (1982).
- Goyal, R. K. & Rattan, S. *Gastroenterology* **74**, 598–618 (1978).
- Hammer, R., Berrie, C. P., Birdsall, N. J. M., Burgen, A. S. V. & Hulme, E. C. *Nature* **283**, 90–92 (1980).
- Arunlakshana, O. & Schild, H. O. *Br. J. Pharmac.* **14**, 48–58 (1959).
- Hirschowitz, B. I. *et al.* (eds) *Subtypes of Muscarinic Receptors* (Elsevier, Amsterdam, 1984).
- Morita, K., North, R. A. & Tokimasa, T. *J. Physiol., Lond.* **333**, 125–139 (1982).
- Surprenant, A. *Trends pharmac. Sci.* (in the press).
- Chassaing, C., Dureng, G., Baissat, J. & Duchene-Marullaz, P. *Life Sci.* **35**, 1739–1745 (1984).
- Brown, H. F. *Physiol. Rev.* **62**, 505–530 (1982).
- Iijima, T., Irisawa, H. & Kameyama, M. *J. Physiol., Lond.* **359**, 485–501 (1985).
- Belluzi, O., Sacchi, O. & Wanke, E. *J. Physiol., Lond.* **358**, 109–129 (1985).
- Kuba, K. & Koketsu, K. *Jap. J. Physiol.* **26**, 703–716 (1976).
- Iwatsuki, N. & Petersen, O. H. *J. Physiol., Lond.* **269**, 735–751 (1977).
- Kuba, K. & Koketsu, K. *Prog. Neurobiol.* **11**, 77–169 (1977).
- Bolton, T. B. *Physiol. Rev.* **59**, 606–718 (1979).
- Kilbinger, H., Halim, S., Lambrecht, G., Weiler, W. & Wessler, I. *Eur. J. Pharmac.* **103**, 313–320 (1984).
- Fuder, H., Rink, D. & Muscholl, E. *Naunyn-Schmiedeberg's Archs Pharmac.* **318**, 210–219 (1982).
- Raiteri, M., Leardi, R. & Marchi, M. *J. Pharmac. exp. Ther.* **228**, 209–214 (1984).
- Mash, D. C., Flynn, D. D. & Potter, L. T. *Science* **228**, 1115–1117 (1985).

Five candidate cDNA clones remained after the sequential screening procedure described in Fig. 1a. To confirm that these cDNA clones encoded acetylcholinesterase, two of them (λ ACHE-1 [insert size 2.3 kilobases (kb)] and λ ACHE-5 [insert size 1.4 kb]) were selected for characterization. Poly(A)⁺ RNA, selected by hybridization with clone λ ACHE-5, directed the translation of at least one peptide of relative molecular mass (M_r) ~ 62,000 (62K) in a rabbit reticulocyte translation system. The polypeptide was selectively immunoprecipitated with antibodies to *Torpedo* acetylcholinesterase and was enriched over total translation products after hybridization selection (not shown). The nucleotide sequence of λ ACHE-5 that is homologous to probes 1 and 2 corresponded exactly to the CNBr peptide used to design the oligonucleotides (Fig. 1b, residues 379–405) as well as to other overlapping tryptic and chymotryptic peptides (residues 358–386). Hybridization and sequence comparison showed that λ ACHE-5 is identical to the 1.4-kb *Eco*RI fragment of λ ACHE-1 (Fig. 1a). As λ ACHE-1 contained the largest insert (2.3 kb) and sequence coding for the amino-terminal region of the peptide, it was selected for complete sequence analysis.

When the amino acids inferred from the λ ACHE-1 sequence are aligned with the peptide sequences (Fig. 1b), the entire coding region for the processed enzyme is defined, starting at nucleotide 41 (residue 1), which corresponds exactly to the N-terminal peptide, and ending at residue 575, which corresponds to the candidate C-terminal tryptic peptide determined from amino-acid sequencing. This portion of the coding region has a calculated M_r of 65,612. The slight difference in size compared with the native catalytic subunits can be attributed to the presence of 7–8% carbohydrate. The amino-acid composition, inferred from the cDNA sequence, is in accord with compositions reported for the native enzyme^{4,9}. The enzyme contains eight cysteines, at least one of which is involved in intersubunit crosslinking.

The region 5' of the amino-terminal residue in the native enzyme (nucleotides 2–40) codes for 13 amino acids which form a portion of the leader peptide characteristic of membrane-associated and exported protein precursors¹⁰. Leader peptides are rich in hydrophobic amino acids and terminate with amino acids containing small neutral side chains¹¹. These criteria are consistent with the sequence in this region, which has hydrophobicity values compatible with a membrane-spanning segment and ends with alanine. Analysis of the hydrophobicity¹² of acetylcholinesterase reveals a profile typical for a globular protein (data not shown). Hydrophilic regions were found between residues 39 and 93 and between residues 535 and 572. Long hydrophobic regions, which are candidates for membrane-spanning segments, were not found; this was expected as the asymmetric form is associated with the basal lamina of the synapse and the globular form contains a post-translational modification whose hydrophobicity probably accounts for its membrane association⁵.

A serine hydroxyl serves as the ester site of the active centre where breakdown of the acetyl-enzyme is rapid. The slower breakdown for phosphoryl esters accounts for their inhibitory properties and the active-site serine can be labelled with ³H-diisopropylfluorophosphate. The position of the active-site serine (residue 200) was identified by aligning the active-centre peptide sequence¹³ with the inferred amino-acid sequence (Fig. 1b, arrow). A search for internal homologies failed to show significant duplication within the amino-acid sequence, although two segments of short length with predominantly negative charges (residues 133–145 and 444–466) showed some similarity. Whether these regions contribute to the peripheral anionic site and the anionic subsite of the active centre must await their assignment by site-directed labelling and analysis of crystal structure.

The catalytic subunits of the asymmetric form of acetylcholinesterase migrate more slowly on SDS-gel electrophoresis

Primary structure of *Torpedo californica* acetylcholinesterase deduced from its cDNA sequence

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Acetylcholinesterase, an essential enzyme of the nervous system, rapidly terminates the action of acetylcholine released into the synapse. Acetylcholinesterase is also found (in lower abundance) in extrajunctional areas of muscle and nerve and on erythrocyte membranes^{1,2}. Hydrodynamic analyses of the native enzyme³ and characterization of its dissociated subunits have revealed multiple enzyme forms which can be divided into two classes: (1) dimensionally asymmetric forms which are usually found within the synapse and contain a collagen-like structural subunit disulphide-linked to the catalytic subunits; and (2) globular forms which appear to be widely distributed on the outer surface of cell membranes. Both forms have been characterized in the ray *Torpedo californica* and, although their catalytic behaviours seem to be identical, they differ slightly in amino-acid composition, peptide maps and reactivity with certain monoclonal antibodies^{4–7}. Here, we report the complete amino-acid sequence of an acetylcholinesterase inferred from the sequence of a complementary DNA clone. The 575-residue protein shows significant homology with the C-terminal portion of thyroglobulin⁸.

Fig. 1 a, Sequencing strategy for cDNA clone λ ACHE-1 encoding acetylcholinesterase from the electric organ of *Torpedo californica*. Restriction endonuclease *EcoRI* fragments from a partial digest of λ ACHE-1 were isolated and either inserted into the sequencing vector M13mp8 (ref. 30) or digested further with *AluI* or *HaeIII* and then cloned. DNA sequencing of the resulting M13-ACHE-1 recombinants was done by the dideoxynucleoside sequencing procedure³¹. Sequencing reactions were primed with either a 17-mer universal primer (Amersham N.4511) (indicated by a vertical line at the beginning of an arrow) or unique 17-mer primers chemically synthesized from a confirmed cDNA sequence (indicated by arrows beginning with a solid circle). Confirmed sequence was obtained from both strands of the cDNA as indicated by arrow length and direction. Sequence data were managed and fragment overlaps determined using computer programs developed by Staden³². Assignment of the correct open reading frame was determined by overlap with the sequence of ~80% of the tryptic peptides and 30% of the CNBr peptides.

b, Primary structure of the *T. californica* acetylcholinesterase. The nucleotide sequence of the mRNA was deduced from the 2,344-base nucleotide sequence of cDNA clone λ ACHE-1. Nucleotides are numbered in the 5' to 3' direction. The predicted amino acids are shown below the corresponding nucleotide sequence, beginning with residue 1 (nucleotide 41), the first amino acid of the mature enzyme, and terminating at position 1,765 with the translation stop codon (TGA), followed by 579 nucleotides of untranslated sequence. Neither the 5' nor the 3' end of the mRNA sequence is complete, given the absence of the initiation codon AUG(Met) at the 5' end, and the polyadenylation signal AATAAA and poly(dA) tract at the 3' end of λ ACHE-1. Underlining indicates some of the peptides used to determine the correct reading frame including the N-terminal peptide, active-centre peptide (the arrow indicates the active-site Ser 200), overlapping tryptic and CNBr peptides used for probe design, and the carboxy-terminal tryptic peptide. A broken line indicates the location of the peptide sequence used for oligonucleotide construction. There are four potential sites of N-linked glycosylation (residues 59, 416, 457 and 533) predicted by the sequence Asn-X-Ser/Thr, where X represents any amino acid except proline³³.

Methods. **a**, A cDNA library (provided by Dr Toni Claudio of Yale University) was constructed from total poly(A)⁺ RNA extracted from the electric organ of *T. californica* and was inserted into the *EcoRI* site of λ gt10. The λ gt10 library (2×10^5 plaque-forming units) was plated out, and nitrocellulose filter copies were prepared³⁴ and screened with two adjacent but not overlapping oligonucleotide probes based on the observed amino-acid sequence of a CNBr peptide isolated from the asymmetric form of the enzyme. Each probe was designed to complement the predicted mRNA sequence as follows. Probe 1, (Met)-Asp-Asp-Asn-Gly-Ile [5'-ATNCC(A,G)TT(A,G)TT(A,G)TC(A,G)TCCAT-3'], a 20-mer with 64-fold degeneracy; probe 2, Asp-Gly-Leu-Asp-Asp-Ile [5'-AT(A,G)-TC(A,G)TCNCA(A,G)NCC(A,G)TC-3'], a 17-mer with 256-fold degeneracy (N = A, G, T or C). Oligonucleotides used for filter hybridization screening were synthesized on a Syntec Microsyn-1450 automated DNA synthesizer using phosphoramidite chemistry³⁵. After ultraviolet shadow-purification (M. Zoller, personal communication), oligonucleotides were 5'-end-labelled with [γ -³²P]ATP ($> 7,000$ Ci mmol⁻¹, Biomedicals, Inc.) using polynucleotide kinase (Amersham). Unreacted [γ -³²P]ATP was removed using NACS prepac columns (BRL), resulting in specific activities of 1×10^5 – 10^6 c.p.m. μ g⁻¹. Conditions of hybridization were essentially those of Wallace *et al.*³⁶, with the addition that the filter copies were washed at successively higher temperatures to optimize the signal-to-background ratio. When 24 candidates selected on the basis of hybridization were rescreened with probes 1 and 2, seven different plaques gave signals greater than the control. A final screening of these candidates provided five plaques which hybridized unambiguously to both probes on duplicate filters. λ DNA was prepared from the final candidate plaques by established methods³⁷. After digestion of clones λ ACHE-1–5 with *EcoRI*, insert sizes were found to range from 1.4 to 2.3 kb. Four inserts contained an internal *EcoRI* site generating a common 0.9-kb fragment plus a fragment in the range 1.2–1.4 kb. A fifth clone contained a single 1.4-kb fragment. Probe 1 hybridized to the 1.2–1.4-kb fragments whereas a probe derived from the amino-terminal protein sequence reacted only with the common 0.9-kb fragment of λ ACHE-1–4.

than the globular form, either as the mature protein or following deglycosylation. Electrophoretic analysis of *in vitro* translation products of *Torpedo* electric organ RNA showed two distinct immunoprecipitable products migrating just slower than the corresponding deglycosylated asymmetric and globular forms (ref. 14 and data not shown). These results indicate that the different forms of acetylcholinesterase may arise from different messenger RNAs. Northern blot hybridization of *Torpedo* electric organ RNA revealed multiple RNA species remaining after

high-stringency washes (Fig. 2); identical results were found when either pACHE-1a or the C-terminal (1.4 kb) fragment of pACHE-5 was used as a probe. The multiple RNA species were enriched in the poly(A)⁺-containing lane and ranged in size from 2.2 to nearly 20 kb. The larger of these species may be due to unprocessed RNA transcripts as both nuclear and cytoplasmic RNAs are present¹⁵. It is plausible that multiple mRNA species are formed, as when size-fractionated RNA from human brain is microinjected into *Xenopus* oocytes, three broadly distributed

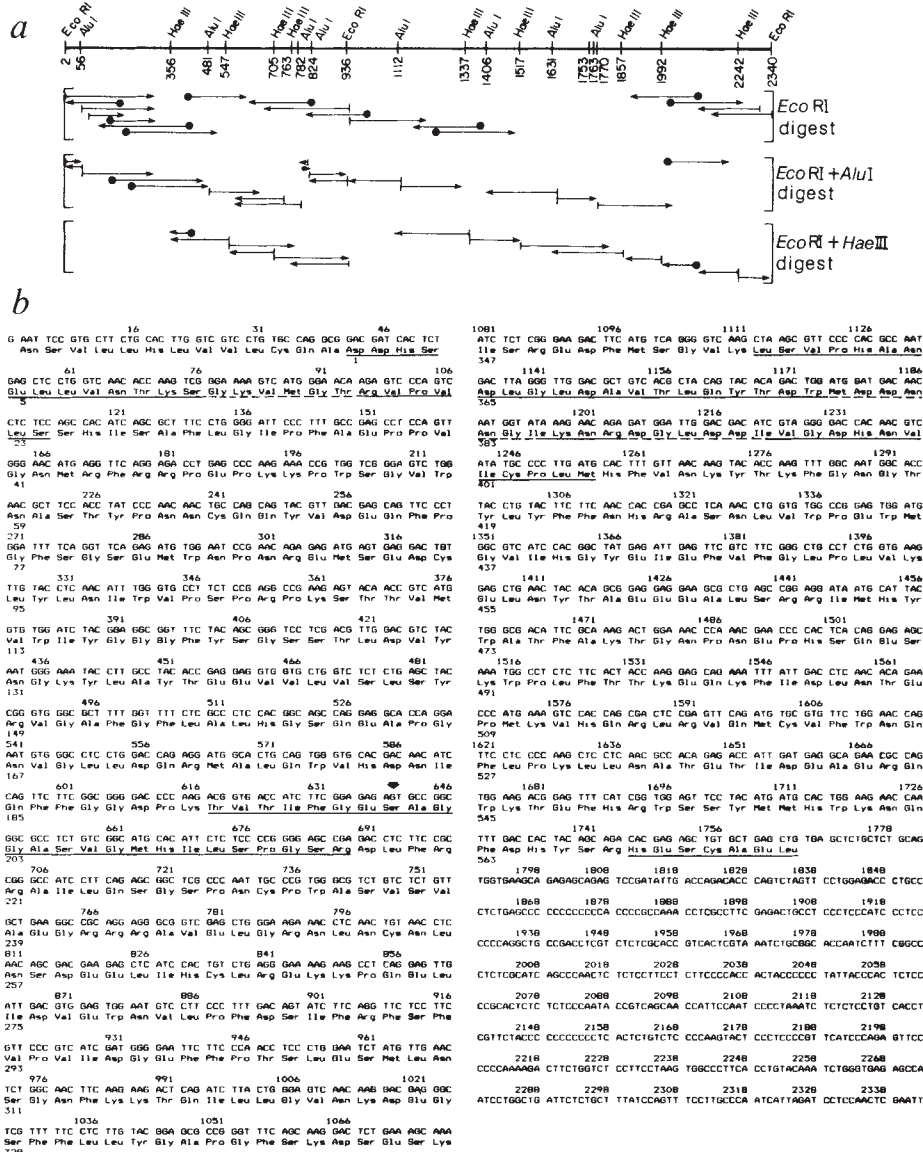
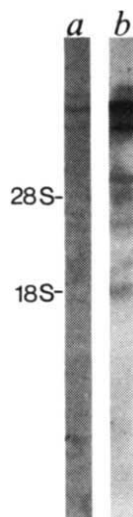


Fig. 2 Blot analysis of *T. californica* electric organ RNA using cDNA probes coding for acetylcholinesterase. Total RNA was prepared by the method of Chirgwin *et al.*¹⁵. Approximately 15 µg of both total (a) and poly(A)⁺-selected (b) RNA were denatured with glyoxal, electrophoresed and transferred to nitrocellulose³⁸. In addition, *Torpedo* ribosomal RNA and *Hind*III fragments of λ DNA were denatured identically and electrophoresed in parallel lanes as standards. Hybridization probes used were: a, pACHE-1a, the N-terminal *Eco*RI fragment of λACHE-1 (nucleotides 2-936) subcloned into the replicative form (RF) of M13mp8; and b, pACHE-5, the C-terminal *Eco*RI fragment (nucleotides 936-2,340) subcloned into M13mp8RF. Both probes were labelled with [α -³²P]dATP (3,000 Ci mmol⁻¹; Amersham) by nick-translation, and used in hybridization at 5 × 10⁶ c.p.m. per lane.



peaks of mRNA induce active cholinesterase¹⁶. Approximately 80% of the tryptic peptides obtained from the catalytic subunit of the asymmetric enzyme have been sequenced (K. MacP.-Q., unpublished results) and these peptides are all found within the sequence inferred from the cDNA clone λACHE-1. Furthermore, the first of two translation stop codons of ACHE-1 appears directly after the sequenced C-terminal tryptic peptide candidate. Hence, the sequence of λACHE-1 probably derives from a cDNA encoding the catalytic subunit of the asymmetric rather than the globular form of the enzyme, although a definitive assignment awaits sequencing of other cDNA clones.

A comparison of the sequence of acetylcholinesterase with proteins found in Newat¹⁷, NBRF and Genbank (BIONET) revealed several interesting features. First, acetylcholinesterase shows neither significant sequence homology in local regions nor global homology with the four nicotinic acetylcholine receptor subunits¹⁸⁻²⁰, despite their similar specificity for inhibitory ligands²¹ and a propensity for both molecules to localize in close apposition at the synapse²². The lack of homology is not unexpected as the dissociation constant (K_D) of acetylcholine for the desensitized state of the receptor is 1.0-3.0 nM^{23,24}, compared with a K_D for acetylcholinesterase of 0.1 mM²⁵. These differences probably reflect receptor recognition of the acetylcholine molecule with its trigonal ester bond, whereas the active centre of acetylcholinesterase can better accommodate a tetrahedral transition state in catalysis²⁶.

Second, although *Torpedo* acetylcholinesterase and human butyrylcholinesterase are homologous in the active-centre region^{13,27}, the two cholinesterases show only limited homology around the active-centre serine with other serine hydrolases; the closest homology was found with aliesterases and carboxyl-esterases. However, no significant homology extending throughout the sequences was found with other serine hydrolases. The serine proteases confer nucleophilicity on the serine through a charge-relay system involving aspartate and histidine residues^{28,29}. In most of these enzymes the positions of the three residues in the overall sequence are conserved. Although acetylcholinesterase contains the active-site serine (residue 200) in a similar position, we did not find a histidine within a potential cysteine loop near residue 57, as would be expected if acetylcholinesterase directly diverged from the pancreatic proteases.

There is a striking homology between acetylcholinesterase starting at residue 30 and the C-terminal domain of bovine thyroglobulin starting at residue 2,210 (ref. 8). Thyroglobulin is a high- M_r dimeric glycoprotein (2 × 330K) that serves as the iodinated precursor of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). Alignment of the 546- and 540-residue segments on the two proteins shows 28% identity, with only 5 gaps in the two peptide sequences. Although the active-site

serine at position 200 is not conserved, the region between residues 144 and 195 in acetylcholinesterase shows 60% residue identity with thyroglobulin. The positions of six of the eight cysteines in the homologous segments are also conserved, which suggests a similar folding pattern in the two proteins. Bovine thyroglobulin is a protein 2,750 amino acids long which contains a C-terminal domain not homologous with other portions of the peptide. As far as is known, acetylcholinesterase, being an enzyme of neurotransmitter degradation, and thyroglobulin, the precursor of T_4 and T_3 , have in common only the functional property of being secreted from specialized cells.

Although the localization and abundance of acetylcholinesterase and the acetylcholine receptor are regulated by synapse formation and muscle activity, acetylcholinesterase alone exhibits extensive structural polymorphism and regulation of distribution of the individual species. The availability of cDNAs for the enzyme should now allow an examination of the regulation of the acetylcholinesterase species at the transcriptional and translational levels.

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- Massoulié, J. & Bon, S. A. *Rev. Neurosci.* **5**, 57-106 (1982).
- Rosenberry, T. L. in *The Enzymes of Biological Membranes* 2nd edn, Vol. 4 (ed. Martonosi, A. N.) 403-429 (Plenum, New York, 1985).
- Massoulié, J. *Eur. J. Biochem.* **11**, 441-455 (1969).
- Lee, S. L., Camp, S. J. & Taylor, P. *J. biol. Chem.* **257**, 12302-12309 (1982).
- Futerman, A. M., Fionini, R. M., Roth, E., Low, M. G. & Silman, I. *Biochem. J.* **226**, 369-377 (1985).
- Lee, S. L., Heinemann, S. & Taylor, P. *J. biol. Chem.* **257**, 12283-12291 (1982).
- Doctor, B. P., Camp, S., Gentry, M. K., Taylor, S. S. & Taylor, P. *Proc. natn. Acad. Sci. U.S.A.* **18**, 5767-5771 (1983).
- Mercken, L., Simons, M. J., Swillens, S., Massaer, M. & Vassart, G. *Nature* **316**, 647-650 (1985).
- Taylor, P. & Jacobs, N. M. *Molec. Pharmacol.* **10**, 78-92 (1974).
- Blöbel, G. & Dobberstein, B. *J. Cell Biol.* **67**, 835-851 (1975).
- Garnier, J., Gaye, P., Mercier, J. C. & Robson, B. *Biochimie* **62**, 231-239 (1980).
- Kyte, J. & Doolittle, R. *J. molec. Biol.* **157**, 105-132 (1982).
- MacPhee-Quigley, K., Taylor, P. & Taylor, S. J. *J. biol. Chem.* **260**, 12185-12189 (1985).
- Sikorav, J. L., Grassi, J. & Bon, S. *Eur. J. Biochem.* **145**, 519-529 (1984).
- Chirgwin, J. M., Przybyla, A. E., McDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1979).
- Soreq, H., Zevin-Sankin, D. & Razon, N. *EMBO J.* **3**, 1371-1375 (1984).
- Doolittle, R. F. *Science* **214**, 149-159 (1981).
- Claudio, T., Ballivet, M., Patrick, J. & Heinemann, S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1111-1115 (1983).
- Noda, M. *et al. Nature* **299**, 793-797 (1982).
- Noda, M. *et al. Nature* **301**, 251-255 (1983).
- Changeux, J. P. *Molec. Pharmacol.* **2**, 369-392 (1966).
- Fallon, J. R., Nitkin, R. M., Reist, N. E., Wallace, B. G. & McMahan, U. J. *Nature* **315**, 571-574 (1985).
- Taylor, P., Brown, R. D. & Johnson, D. A. *Curr. Topics Membranes Transport* **18**, 407-444 (1983).
- Changeux, J.-P., Devillers-Thiery, A. & Chemouilli, P. *Science* **225**, 1335-1345 (1984).
- Rosenberry, T. L. *Adv. Enzym. Relat. Areas molec. Biol.* **43**, 102-218 (1975).
- Taylor, P. & Jacobs, N. M. *Molec. Pharmacol.* **10**, 93-107 (1974).
- Lockridge, O. in *Cholinesterases: Fundamental and Applied Aspects* (eds Brzin, M., Barnard, E. A. & Sket, D.) 5-11 (Walter de Gruyter, Berlin, 1984).
- Hartley, B. S. *Phil. Trans. R. Soc. B257*, 77-87 (1970).
- Davie, E. W., Fujikawa, K., Kurachi, K. & Kiesel, W. *Adv. Enzym.* **48**, 277-318 (1979).
- Messing, J. *Meth. Enzym.* **101**, 20-78 (1983).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Staden, R. *Nucleic Acids Res.* **8**, 3673-3694 (1980).
- Bause, E. *Biochem. J.* **209**, 331-336 (1983).
- Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).
- Sinha, N. D., Biernat, J., McManus, J. & Koster, H. *Nucleic Acids Res.* **12**, 4539-4557 (1984).
- Wallace, R. B. *et al. Nucleic Acids Res.* **9**, 879-894 (1981).
- Davis, R. W., Botstein, D. & Roth, J. R. *Advanced Bacterial Genetics*, 70-82 (Cold Spring Harbor Laboratory, New York, 1980).
- Thomas, P. S. *Meth. Enzym.* **100**, 255-266 (1983).

Single channels and ionic currents in peptidergic nerve terminals

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Control of secretion, by a mechanism in which membrane depolarization leads to Ca^{2+} entry¹, has been extensively studied. The small size and inaccessibility of most nerve terminals, however, have precluded direct analysis of membrane ionic currents and their influence on secretion (with some notable exceptions^{2,3}). Recently, patch-clamp methods have been applied to several secretory systems⁴⁻⁶ for both voltage-clamp and single-channel recordings. We now report the extension of this analysis to isolated peptidergic nerve terminals. We used terminals obtained from a crustacean neurohaemal organ, the sinus gland. Analyses of currents under whole-terminal voltage clamp showed inward currents carried by Na^+ and by Ca^{2+} , and outward currents carried predominantly by K^+ . Furthermore, we have observed two types of single-channel currents that may be unique to nerve terminals. Both show little selectivity between Na^+ and K^+ . The first channel is activated by intracellular Na^+ and the second by intracellular Ca^{2+} . These channels have conductances of 69 and 213 pS, respectively, in symmetrical 310 mM KCl. It should now be possible to compare electrical activity recorded intracellularly from intact nerve endings^{7,8}, with whole-cell and single-channel currents and with the release of peptide neurohormones from isolated neuronal terminals.

Sinus gland terminals from the land crab, *Cardisoma carnifex*, are easily isolated after treatment with collagenase (see Fig. 1). In electron micrographs the isolated nerve endings appear as circular profiles densely packed with neurosecretory granules (Fig. 1C). Dissociated neuronal terminals are morphologically very similar to non-dissociated nerve endings *in situ* (compare with Fig. 1A). The same morphologically distinguishable terminal types as those observed *in situ*⁹ can be recognized among the isolated terminals (Fig. 1C). A single *C. carnifex* sinus gland yields hundreds of isolated terminals, many of diameter $>10\ \mu\text{m}$ (Fig. 1B). The terminals readily form seals of $>10\ \text{G}\Omega$ with fire-polished electrodes, making feasible the application of patch-clamp techniques¹⁰ in order to characterize the ionic currents and channels of the nerve terminal membrane. Dissociated nerve terminals exhibit resting potentials between -30 and $-50\ \text{mV}$ (intact nerve endings have values of -50 to $-60\ \text{mV}$ ⁸) and spontaneous overshooting action potentials. These and other observations, reported below, show that the nerve terminals, after dissociation, are viable and comparable to nerve endings *in situ*.

Currents in isolated nerve terminals were recorded under voltage clamp by 'whole-terminal recording' in analogy with whole-cell recording¹⁰. In response to depolarizing voltage commands, the initial current was inward, followed by outward, maintained current (Fig. 2A). In some nerve terminals, the inward current has the properties expected of a Ca current¹¹: it is not blocked by tetrodotoxin (TTX) [Fig. 2A(ii)], it is reduced by external application of Cd^{2+} [Fig. 2(iii)], and it begins to 'run down' after 30–40 min. The Ca current relaxes more slowly in certain terminals, which could explain observed differences in the release pattern. Release of red-pigment-concentrating hormone¹² is brief and is associated with a voltage-dependent Ca entry which inactivates¹³, while the release of other peptides

is more prolonged and is probably associated with non-inactivating Ca entry⁸. In some terminals a Cd^{2+} -resistant component [Fig. 2B(ii)] was blocked by TTX [Fig. 2B(iii)]. Thus, there are two inward currents: one carried by Na^+ and the other by Ca^{2+} . This was expected as previous studies showed that most crab nerve endings exhibit overshooting action potentials having both Na^+ and Ca^{2+} components^{7,8,14}. Outward currents were partially reduced by the application of tetraethylammonium (TEA; Fig. 2A) (consistent with an increase by TEA of the duration of the action potential in intact terminals¹⁴) and totally blocked if the terminal was internally perfused with Cs^+ (Fig. 2B). These results indicate that the outward currents are carried by K^+ .

Single-channel currents were recorded from terminals in cell-attached and inside-out patches with 310 mM KCl on both sides of the patch. Two types of ionic channels—possibly unique to nerve terminals—were observed: (1) the f channel (for 'first') shows brief (millisecond) transitions to the open state, sometimes occurring in bursts, with long (seconds) intervals between openings [Fig. 3A(i)]. This channel, in symmetrical K^+ ($\square$), has a mean slope conductance of $69 \pm 3.6\ \text{pS}$ ($n=6$) (Fig. 3C). The single-channel I - V curve is not changed by substitution of K_2SO_4 (data not shown) or Na_2SO_4 ($\blacktriangle$) for KCl on the external (or internal) face of the patch, but shows rectification when CsCl ($\bullet$) is substituted, indicating failure of the channel to pass Cs^+ or Cl^- (Fig. 3C). Channel currents in the presence of a salt gradient show nearly perfect selectivity for cations as opposed to anions (see, for example, Fig. 3A). With a fourfold salt gradient (external NaCl concentration = 310 mM, internal NaCl concentration = 78 mM; osmolarity preserved with sucrose) we found that these currents reversed at $+35\ \text{mV}$, as would be expected for a perfectly cation-selective channel. These two observations argue against a significant anion permeability of the channels. Na^+ passes through the channel just as easily as K^+ because the reversal potential with equal concentrations of KCl externally and NaCl internally remained at 0 mV (data not shown). Furthermore, this (420 mM) and lower (for example, 78 mM) concentrations of Na^+ on the inside of the patch, caused this type of cation channel to remain open for longer periods [Fig. 3A(ii)] on depolarization. This channel can be observed in solutions having the internal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) buffered to $10^{-8}\ \text{M}$ with EGTA.

(2) The other type of channel (s, for 'second') exhibits much longer (seconds) openings (Fig. 3B) and has a mean conductance (Fig. 3D) of $213 \pm 6.1\ \text{pS}$ ($n=4$) in symmetrical K^+ ($\square$). The openings seem to occur in bursts with rapid flickering back to the closed state. This channel type is rarely observed in solutions having low $[\text{Ca}^{2+}]_i$ (data not shown), except during large voltage steps ($+80\ \text{mV}$), but is activated by increasing the internal free Ca^{2+} concentration to $>1\ \mu\text{M}$ (see Fig. 3B). Higher concentrations of Ca^{2+} cause a transient activation of channel activity. Ion substitution experiments indicate that this channel also has nearly equal permeabilities for Na^+ ($\triangle$) and K^+ ($\square$), but, unlike the f channel, it allows Cs^+ ($\bullet$) to pass through (Fig. 3D). Inward current events having the characteristics of this large channel can be observed in whole-terminal recordings, under voltage clamp, with crab saline¹⁵ in the bath. The s channel, therefore, has the characteristics of a Ca -activated cation channel¹⁶, but exhibits a much larger slope conductance than has been observed in other cells (22 – $38\ \text{pS}$ ^{6,16,17}). This channel could be termed the 'maxi' Ca -activated cation channel, in analogy with Ca -activated K^+ channels¹⁸. The f channel is seen in almost every patch, but the other, larger s channel occurs less frequently; this could be caused by our use of low Ca^{2+} concentrations to achieve the inside-out configuration.

One could conceive of a scheme by which Na^+ and/or Ca^{2+} entry activates the channel(s), which then becomes inactivated by Ca^{2+} after a period of seconds. These channels could be responsible for the long-lasting plateau potentials which underlie bursting in these terminals^{7,19}. Burst patterns lead to the potentiation of hormone release, for example, in the isolated

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Fig. 1 **A**, Electron micrograph of intact sinus gland showing the dense aggregation of terminals and of neurosecretory granules within terminal profiles. Note the large size (5–30 μm diameter) of the terminals, the paucity of non-terminal structures, and the close apposition of the terminals to epineurium and blood sinus (where the scale bar = 1 μm) is located). **B**, Phase-contrast micrograph of dissociated nerve terminals after isolation for patch-clamping experiments. Two large terminals (~25 μm in diameter) can be seen in this field. Note the variety of sizes (corresponding to *in situ* distribution), paucity of non-terminal material, and abundance of terminal structures. **C**, Electron micrograph of isolated nerve endings. Note that the nerve terminals are membrane-delimited and still densely packed with neurosecretory granules; they are comparable to nerve endings in the intact sinus gland (see **A**).

Methods. Isolation of peptidergic neurosecretory terminals. The sinus gland was exposed to collagenase (1 mg ml⁻¹) for 30 min at 32 °C (this treatment does not affect channel activity, but does lead to better seals on the terminal membrane), rinsed, dissociated by drawing the gland in and out of a pipette tip in an 'intracellular-type' medium [(in mM): 300 KCl, 15 NaCl, 2 EGTA, 100 HEPES (pH 7.2), 2 MgCl₂, 0.3 CaCl₂, 2 ATP, 530 sucrose, giving a free Ca²⁺ concentration of 10 nM] and then returned to crab saline¹⁵ containing 5 mM glucose. Care was taken when changing solutions so that the terminals were never exposed to conditions where release would occur (that is, elevated [K⁺] with [Ca²⁺] greater than 100 nM). The isolated nerve endings were then attached to the bottom of a plastic dish which had been treated previously with 1% protamine sulphate. For electron microscopy, the nerve terminals were centrifuged, fixed with 4% glutaraldehyde in 0.1 M cacodylate pH 7.0, containing 400 mM sucrose, and post-fixed with 1% OsO₄.

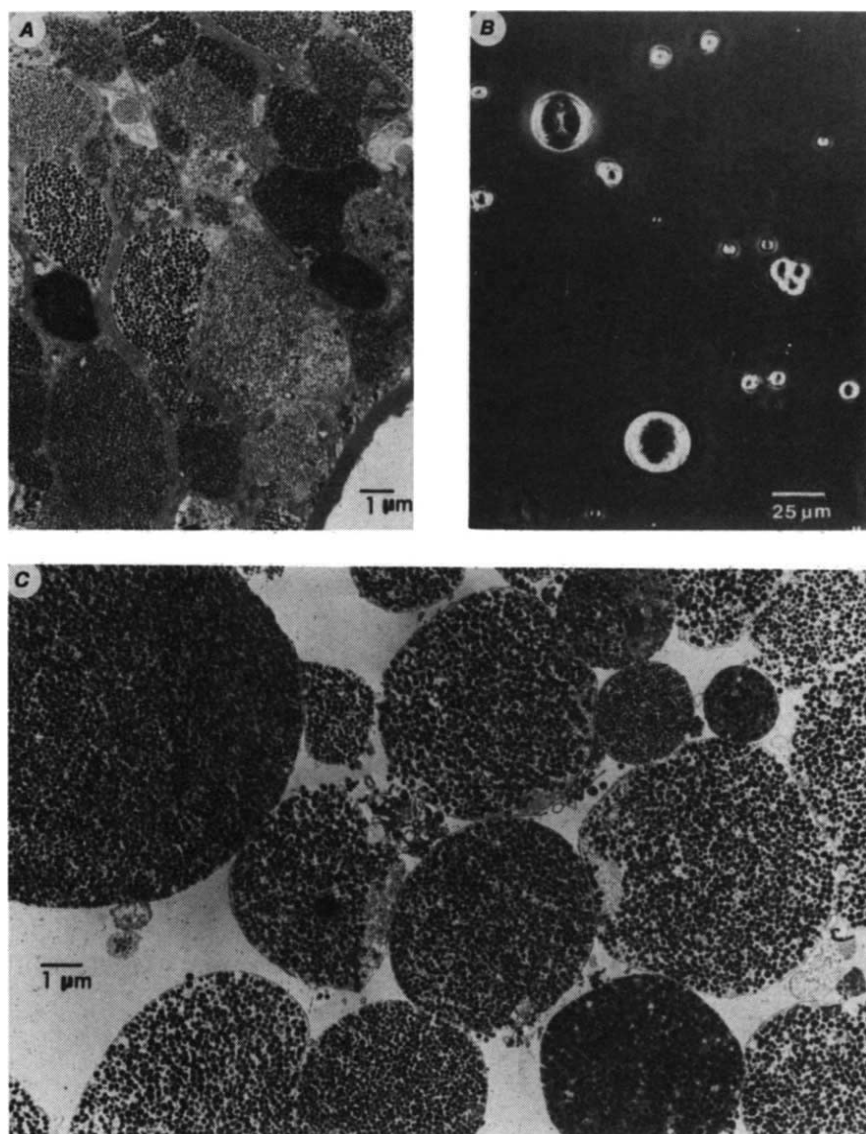
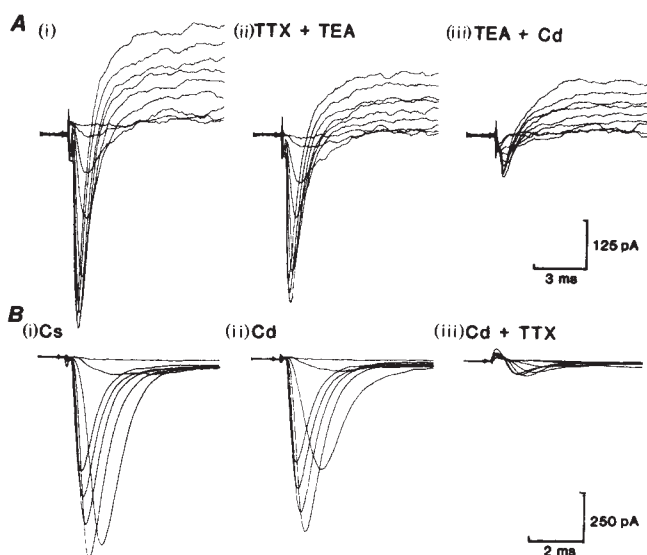


Fig. 2 Activation of whole-cell currents in dissociated sinus gland nerve terminal during depolarizing voltage steps from a holding potential equal to the normal resting potential (−50 mV). Note that the magnitude of the currents is directly related to the size of the terminal (compare **A** with **B**), as might be expected from differences in their membrane surface area. All records are leak-subtracted. **A(i)**, Appearance of both inward and outward currents during clamp steps to membrane potentials of −25 mV to +15 mV, incremented by +5 mV each time; 10 s between steps. Isolated nerve ending of 10 μm diameter. (ii) Effect of TEA. Same protocol as for **A(i)**. Note the partial reduction of the inward currents in 6 $\times 10^{-7}$ M TTX. (iii) Effect of Cd²⁺ on inward currents. Perfusion with 1 mM Cd²⁺ of the same terminal (but in 50 mM TEA) caused a marked reduction of the inward currents. **B(i)**, Effect of Cs⁺ on outward currents in a different nerve terminal (15 μm diameter). Same protocol as in **A**, except that steps were from −25 mV to +35 mV in 10 mV increments, and K⁺ was replaced by Cs⁺ in the patch pipette, resulting in an absence of outward currents [compare with **A(i)**]. (ii) 1 mM Cd²⁺ had little effect on inward currents in this terminal. Same protocol as for **B(i)**. (iii) Perfusion of 6 $\times 10^{-7}$ M TTX blocked residual inward current in this nerve ending. **Methods.** Standard patch-clamp recording techniques¹⁰ were used to attain whole-cell configurations with seal resistances of >10 G Ω from dissociated nerve terminals of the sinus gland. The nerve endings were perfused with crab saline¹⁵ externally and the pipette contained 'intracellular-type' medium (see Fig. 1 legend). In **B**, 315 mM CsCl replaced both KCl and NaCl in the patch pipette. All solutions were made osmotically equivalent by either reducing the NaCl concentration or adding sucrose.



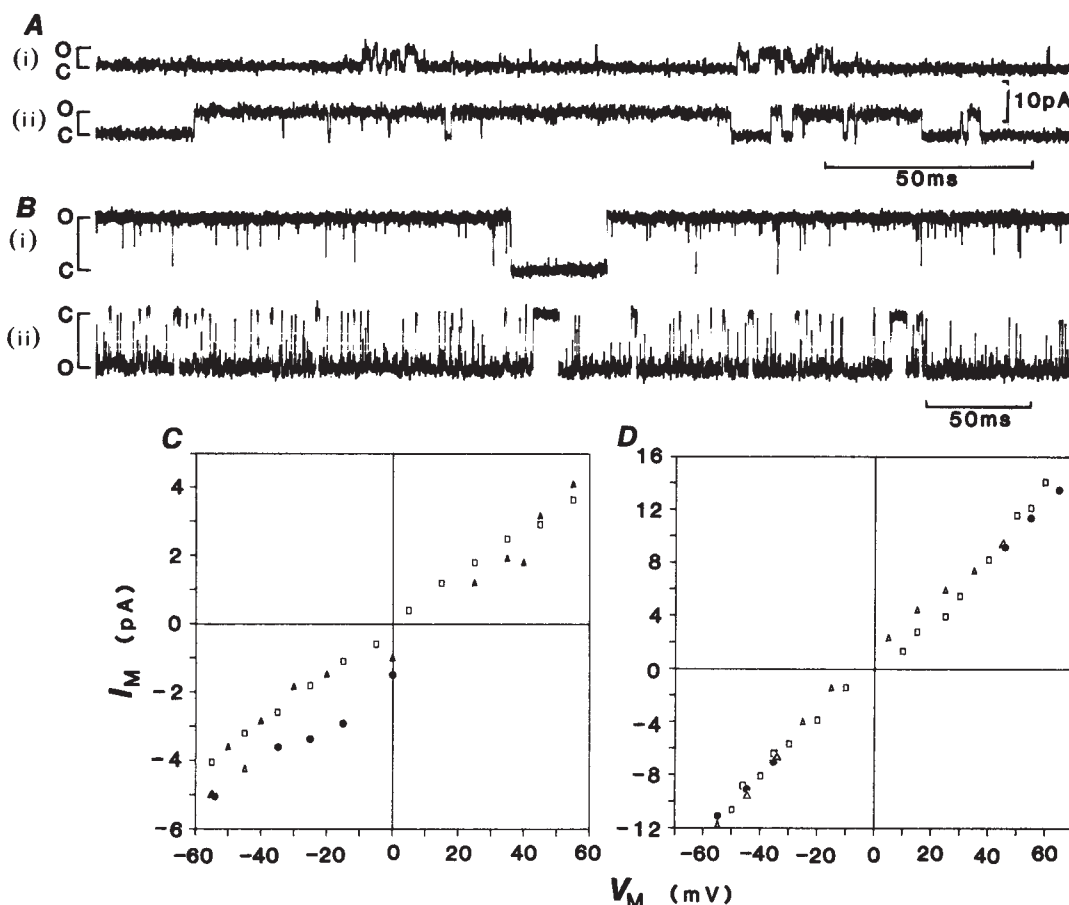


Fig. 3 Single-channel currents from inside-out patches showing two types of cation channels observed in isolated nerve terminals. **A**, First ('f') channel type: (i) patch held at +55 mV in symmetrical KCl. Note the bursts of channel openings. Upward openings (O) indicate outward current. (ii) Same patch held at +45 mV with 310 mM KCl outside and 210 mM Na₂SO₄ inside. Note the increased duration of the openings. Similar effects were seen with 78 mM NaCl (comparable to concentrations of Na⁺ which activate a cardiac outward current²³) on the inside of the patch. The similar amplitude as at +55 mV (i) is the result of a shift in the *I*-*V* relationship due to the asymmetrical salt gradient and is in the direction predicted by the Nernst equation for a cation-selective channel. The increased duration of openings is not caused by a difference in holding potential. **B**, Second ('s') channel type: (i) patch held at +55 mV with 310 mM KCl inside and 210 mM Na₂SO₄ outside, and with 3 μM [Ca²⁺]_i. (ii) Same patch as (i) but held at -40 mV. Note the similar amplitude as that obtained at +55 mV, resulting from a shift in the *I*-*V* relationship due to the asymmetrical salt gradient, again in the direction predicted for a cation-selective channel. Channel openings are rarely, if ever, seen at these potentials if the [Ca²⁺]_i is less than 10⁻⁶ M. Downward openings indicate inward current. All records are at the same gain to emphasize the difference in size of the two channel types. **C**, *I*-*V* relationship of the f channel. Channel openings of small amplitude (*I*_M) and short duration (see **A**) were plotted against patch potential (*V*_M). The calculated regression has a slope conductance of 69 pS (*r* = 0.99; *n* = 6) in symmetrical KCl (□). Note that there was little change in conductance when KCl in the external solution was replaced by 210 mM Na₂SO₄ (▲), but rectification occurred when it was replaced in the internal solution by 310 mM CsCl (●). **D**, *I*-*V* relationship of the s channel. Channel openings of larger amplitude and longer duration (see **B**) were plotted against patch potential. The calculated regression has a slope conductance of 213 pS (*r* = 0.95; *n* = 4) in symmetrical KCl (□). Note that there was little change in conductance when KCl in the internal solution was replaced by either 210 mM Na₂SO₄ (Δ) or 310 mM CsCl (●).

Methods. Standard patch-clamp recording techniques¹⁰ were used to form 'inside-out' patches with seal resistances of >10 GΩ from dissociated nerve terminals of the sinus gland. Records in **A** and **B** were filtered at 4 kHz with 8-pole Bessel characteristics. In **C** and **D** mean channel current values, with symmetrical solutions (310 mM KCl, 2 mM MgCl₂, 2 mM ATP, 485 mM sucrose, 100 nM free Ca²⁺ buffered with 2 mM EGTA, 100 mM HEPES pH 7.2) on both sides of the patch (□) or substitutions as outlined above, are plotted.

neurohypophysis^{20,21}. These two types of channels may also help to explain Na enhancement of transmitter release at crustacean neuromuscular junctions²².

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- Douglas, W. W. *Br. J. Pharmac.* **34**, 451-474 (1968).
- Katz, B. & Miledi, F. *J. Physiol., Lond.* **192**, 407-436 (1967).
- Llinas, R., Steinberg, I. Z. & Walton, K. *Biophys. J.* **33**, 289-322 (1981).
- Fenwick, E. M., Marty, A. & Neher, E. *J. Physiol., Lond.* **331**, 557-597 (1982).
- Kidokoro, Y. in *The Electrophysiology of the Secretory Cell* (eds Poisner, A. M. & Trifaro, J. M.) 195-218 (Elsevier, New York, 1985).
- Maruyama, Y. & Petersen, O. H. *Nature* **299**, 159-161 (1982).
- Cooke, I. M. *J. exp. Biol.* **118** (in the press).
- Stuenkel, E. L. *J. Physiol., Lond.* **359**, 163-187 (1985).
- Weatherby, T. M. *Cell Tissue Res.* **220**, 293-312 (1981).
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. *Pflügers Arch. ges. Physiol.* **391**, 85-100 (1981).
- Hagiwara, S. & Byerly, L. A. *Rev. Neurosci.* **4**, 69-125 (1981).
- Fernlund, P. & Josefsson, L. *Science* **177**, 173-175 (1972).
- Cooke, I. M. & Haylett, B. A. *J. exp. Biol.* **113**, 289-321 (1984).
- Nagano, M. & Cooke, I. M. in *Neurosecretion: Molecules, Cells, Systems* (eds Farmer, D. & Lederis, K.) 501-502 (Plenum, New York, 1981).
- Pantin, C. F. A. *Notes on Microscopical Technique for Zoologist* (Cambridge University Press, 1948).
- Colquhoun, D., Neher, E., Reuter, H. & Stevens, C. F. *Nature* **294**, 752-754 (1981).
- Yellen, G. *Nature* **296**, 357-359 (1982).
- Latorre, R. & Miller, C. J. *Membrane Biol.* **71**, 11-30 (1983).
- Cooke, I. M. & Stuenkel, E. L. in *The Electrophysiology of the Secretory Cell* (eds Poisner, A. M. & Trifaro, J. M.) 115-164 (Elsevier, New York, 1985).
- Dutton, A. & Dyball, R. E. J. *J. Physiol., Lond.* **348**, 601-613 (1984).
- Cazalis, M., Dayanithi, G. & Nordmann, J. J. *J. Physiol., Lond.* **369**, 45-60 (1985).
- Atwood, H. L., Charlton, M. P. & Thompson, C. S. *J. Physiol., Lond.* **335**, 179-195 (1983).
- Kameyama, M. *et al. Nature* **309**, 354-356 (1984).

Neonatal T-cell tolerance to minimal immunogenic peptides is caused by clonal inactivation

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The mechanisms underlying T-lymphocyte tolerance induced in neonatal mice are still unknown¹⁻³. It is unclear whether the tolerant state is the result of inactivation of T cells on exposure to antigen during development or of active suppression by other T cells specific for the same antigen. To distinguish between these two hypotheses, we have analysed the specificity of tolerance to three cytochrome peptides which differ by only a single amino-acid substitution in the epitope recognized by proliferative T cells. The peptides stimulate proliferative responses which are highly specific with minimal cross-reactivity. As antigen-induced clonal inactivation would address the same cells normally activated by that antigen, the specificity of tolerance should exactly match that of the proliferative response to the antigen, and each cytochrome peptide should induce tolerance to itself alone. Conversely, as T-suppressor (T_s) and T-proliferative (T_p) cells almost invariably seem to recognize distinct, non-overlapping determinants on protein antigens (see, for example, refs 4-6), suppressor-mediated tolerance should not be affected by substitutions in the proliferative T-cell epitope. Tolerance would depend solely on the existence of a shared suppressor determinant, so each cytochrome peptide should induce cross-tolerance to the others. We found that the specificity of tolerance matched that of the proliferative response: each peptide induced tolerance for itself but the response to the variants was unaltered. This result strongly supports the hypothesis of clonal inactivation as an important mechanism in induction of neonatal tolerance.

Three peptides were used in this study: K99, which corresponds to amino acids 93-103 of moth cytochrome *c*, and two variants with glutamine or arginine substituted for lysine at residue 99 (Q99 and R99 respectively). These peptides are close to the minimal size for induction of a proliferative response⁷; further shortening of this 11-amino-acid C-terminal peptide of cytochrome *c* substantially reduces its ability to stimulate a response⁸. Furthermore, these peptides were chosen because amino-acid residue 99 has been shown to form a critical part of the major proliferative T-cell epitope on cytochrome *c*^{8,9}, and substitutions at this site radically alter the composition of the responding population. Thus, lymph node cells from adult B10.A mice which had been immunized with K99, R99 or Q99 gave *in vitro* proliferative responses that were highly specific with little cross-reactivity between the peptides (Fig. 1).

Tolerance to each peptide was induced in neonatal B10.A mice by two injections of peptide at 24 and 72 h after birth and the specificity of tolerance was tested in each group by challenge with the peptides at 8-10 weeks. Figure 2a shows the responses of K99-tolerant mice to K99, Q99 and R99. Neonatal administration of K99 induced essentially complete tolerance to itself with only a minimal response even at the highest *in vitro* concentration of the peptide. However, these K99-tolerant mice (broken lines in Fig. 2a) responded normally on immunization with the other peptides, Q99 and R99, compared with the control responses (solid lines). Figure 2b,c shows the results of reciprocal experiments in the Q99- and R99-tolerant groups. The results were comparable: tolerance was specific for the peptide used for its induction and the response to the other peptides was unaffected. Thus, the specificity of tolerance is dependent on substitutions in the proliferative T-cell epitope,

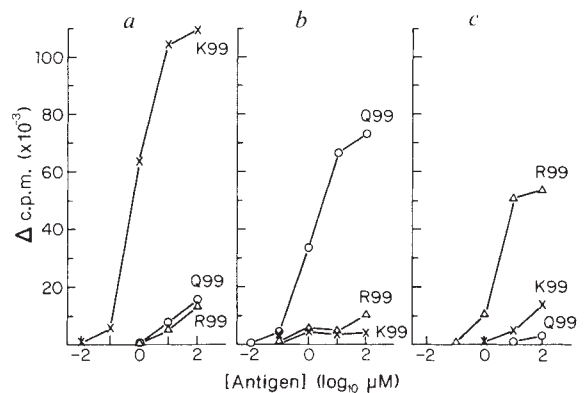


Fig. 1 Specificity profiles of T cells induced by synthetic cytochrome peptides in B10.A mice. The mice were immunized with K99 (a), Q99 (b) or R99 (c). The peptides were prepared by solid-phase peptide synthesis and purified by reverse-phase HPLC using a preparative C_8 column and a solvent system of 0.1% trifluoroacetic acid with an increasing gradient of acetonitrile. Peptide K99 corresponds to the 11 carboxy-terminal amino acids of moth cytochrome *c*, amino acids 93-103 (NH_2 -ELIAYLKQATK-COOH). Q99 and R99 are variants with glutamine or arginine substituted for lysine at residue 99.

Methods. B10.A mice (8-10 weeks old) were immunized with 5 μ g of peptide in saline in a 1:1 emulsion with complete Freund's adjuvant (CFA; Difco) in the hind footpads. After 10 days, popliteal and inguinal lymph nodes were removed and cells from each group (three mice) were pooled. The lymph node cells were washed twice in balanced salt solution and added to 96-well plates at 4×10^5 per well in Click's medium supplemented with penicillin/streptomycin, glutamine, sodium pyruvate, 2-mercaptoethanol and 0.5% normal mouse serum, as well as a range of *in vitro* antigen concentrations (0.01-100 μ M). The cultures were incubated for 5 days in 2% CO_2 , 98% air at 37°C and 1 μ Ci 3H -thymidine (ICN 27 Ci $mmol^{-1}$) was added for the last 18 h. The cells were collected onto glass fibre sheets and incorporation of labelled thymidine was measured by scintillation counting. All cultures were performed in triplicate and the results shown are the arithmetic mean c.p.m. per culture with the background (medium alone) subtracted. The standard errors of the mean were all less than 10,000 c.p.m. and have been omitted for clarity. (27 Ci $mmol^{-1}$; International Chemical & Nuclear.)

and the absence of cross-tolerance between peptides matches the lack of cross-reactivity in the normal response. These data support the hypothesis of clonal inactivation, and exclude a mechanism of suppression by cells recognizing an epitope distinct from the proliferative epitope, which would cause tolerance to be cross-reactive.

Although, *a priori*, it may seem unlikely that a T_s -inducing and a T_p -inducing determinant could coexist on an 11-amino-acid peptide, the possibility of an overlapping suppressor cell epitope should be considered. As the tolerance we have described is peptide-specific, if T_s were involved, they too would have to be non-cross-reactive and recognize an epitope including the unique residue 99. Such suppressor cells could not interact with their targets directly via an antigen bridge but could act by secretion of nonspecific suppressor factors. Functionally, this suppression would be antigen-specific despite the nonspecific effector mechanism as, for example, K99-specific T_s would be activated only by K99 and not by Q99 or R99. This hypothesis was tested by co-immunization of tolerant mice with a mixture of two peptides, one of which had been used to induce tolerance. Clonal inactivation of the T cells specific to one peptide should not affect the response to a second peptide. However, suppression which was nonspecific in the effector phase would inhibit the response to both peptides equally. The experiment shown in Fig. 3 demonstrates that K99-tolerant mice that were co-immunized with a mixture of K99 and R99 gave a nearly normal response to R99. The difference between the normal R99 response and the R99 response in K99-tolerant mice can be

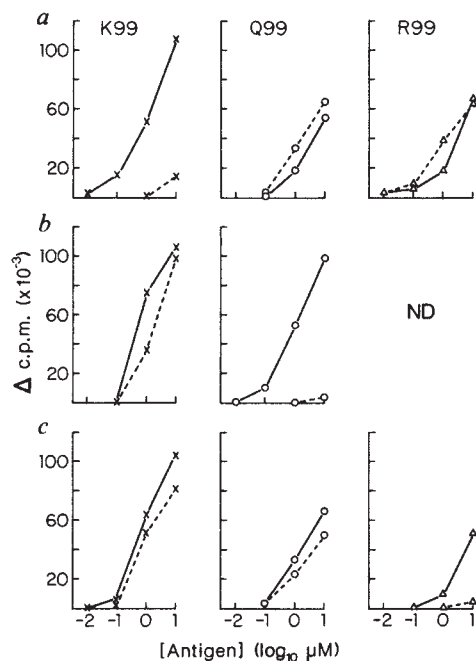


Fig. 2 The specificity of tolerance induced in neonatal mice. Tolerance was induced in neonatal B10.A mice by a method similar to that described previously¹⁰. Peptide (20 μ g) in incomplete Freund's adjuvant (Gibco) was injected into the peritoneal cavity at 24 and 72 h after birth. The mice were immunized at 8–10 weeks of age and their responses measured in an *in vitro* proliferation assay as described in Fig. 1 legend. *a*, The proliferative response of K99-tolerant mice (broken line) compared with normal age- and sex-matched B10.A mice (solid line), and immunization with (from left to right) K99, Q99 or R99. The same peptide as used for immunization was added to the *in vitro* cultures at concentrations from 0.01 μ M to 10 μ M. *b*, *c*, The response of Q99- and R99-tolerant mice, respectively, compared with that of normal controls. ND, not determined.

explained by the loss of the small K99/R99 cross-reactive population seen in Fig. 1. These results exclude the possibility of T_s cells acting by a nonspecific effector mechanism.

To obtain direct evidence for possible active suppression, tolerant cells were mixed *in vitro* with normal, primed cells. The ability of T_s to prevent a proliferative response has been demonstrated in several systems^{10–12}. Lymphocytes from tolerant mice or from complete Freund's adjuvant (CFA)/saline-primed mice were added to the responsive T cells (Fig. 4), but there was no difference between the groups. No indication of suppression was seen over a wide range of cell-cell ratios. Even when tolerant cells constituted a large proportion of the cells in culture, and the number of responsive proliferative cells was limiting, no suppression was observed. Previous work has shown in adult-induced tolerance to hen egg-white lysozyme (HEL), that *in vitro* suppression of the plaque-forming cell response can be masked by other regulatory T cells and can be revealed by removing $Lyt\ 1^{+}2^{+}$ cells from the tolerant lymph node population (A.O. and E.E.S., unpublished). However, treatment of lymphocytes from cytochrome c-tolerant mice, using the same protocol, failed to unmask suppression (data not shown).

The results presented here strongly support clonal inactivation as an important mechanism in induction of neonatal tolerance. In the case of tolerance to minimal immunogenic cytochrome peptides, it appears to be the only mechanism involved. Both antigen-bridging suppression, which is responsible for tolerance to native HEL in adult B10.A mice¹³, and suppression by non-specific suppressor factors, have been excluded in the present study. However, suppression by T_s which directly recognize idiotopes on the proliferative T cells cannot be eliminated, although we consider this unlikely, especially as there was no

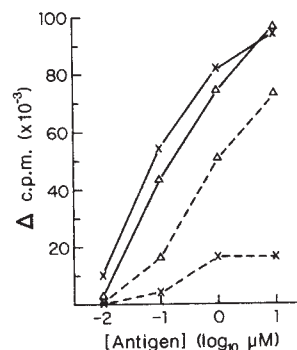


Fig. 3 The response of K99-tolerant mice after co-immunization with a mixture of K99 and R99 peptides. Tolerance was induced to K99 as described in Fig. 2 legend, and the mice were immunized at 8 weeks old with a mixture of K99 and R99 (5 μ g of each) in CFA. The proliferative response of tolerant (broken lines) and age- and sex-matched normal mice (solid lines) to each peptide was assayed *in vitro*. Δ , Response to R99; $\times$, response to K99.

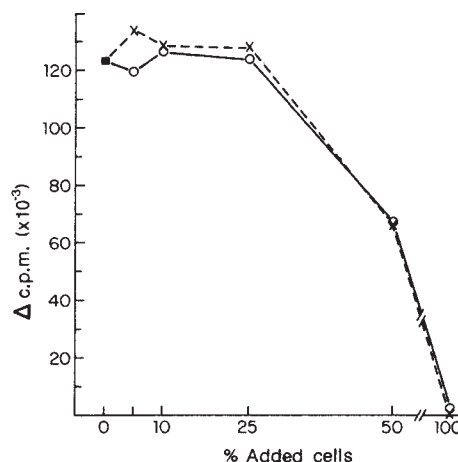


Fig. 4 Effect of the addition of lymph node cells from tolerant mice to normal primed cells, on the K99 proliferative response. K99-tolerant B10.A mice were challenged with K99 and the pooled unfractionated, tolerant lymph node cells ($\times$ --- $\times$) were added in increasing proportions to the cells from normal primed mice. The immunization and culture conditions were as described in Fig. 1 legend. The control wells contained equivalent numbers of lymph node cells from CFA/saline-primed B10.A mice added to the responsive cells ($\circ$ --- $\circ$). The number of cells per well was kept constant at 4×10^5 , and the *in vitro* antigen used was K99 (10 μ M).

evidence of suppression on mixing tolerant and normal responding cells. The failure to demonstrate suppression using these minimal immunogenic peptides is not inconsistent with reports of suppressor cell induction by neonatal administration of native proteins^{3,14}; instead, it supports the concept that the T_s - and T_p -inducing determinants on proteins are inherently different and even in the neonatal milieu, T_p -inducing determinants do not activate T_s . One would expect that a small peptide which bears a suppressor determinant would induce T_s in neonatal mice and subsequently suppress the response to the whole molecule. Thus, both mechanisms—clonal inactivation and suppression—could have a complementary role in induction and maintenance of tolerance. We propose that clonal inactivation is a primary mechanism of T-cell tolerance to proteins and the only mechanism involved in tolerance to minimal immunogenic peptides.

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1. Nossal, G. J. V. *A. Rev. Immun.* **1**, 33-62 (1983).
2. Weigle, W. O. *Adv. Immun.* **30**, 159-273 (1980).
3. Loblay, R. H., Fazekas de St Groth, B., Pritchard-Briscoe, H. & Basten, A. J. *exp. Med.* **157**, 957-973 (1983).
4. Adorini, L., Harvey, M. A., Miller, A. & Sercarz, E. E. *J. exp. Med.* **150**, 293-306 (1979).
5. Kryzch, U., Fowler, A. & Sercarz, E. E. in *Protein Conformation as an Immunological Signal* (eds Celada, F., Schumaker, N. & Sercarz, E. E.) 395-408 (Plenum, London, 1983).
6. Swanborg, R. H. *J. Immun.* **114**, 191-194 (1975).
7. Thomas, D. W., Meltz, D. S. K. & Wilner, G. D. *J. Immun.* **123**, 759-764 (1979).
8. Hansburg, D., Fairwell, T., Schwartz, R. H. & Appella, E. *J. Immun.* **131**, 319-328 (1983).
9. Schwartz, R. H. *A. Rev. Immun.* **3**, 237-261 (1985).
10. Araneo, B. A. & Kapp, J. A. *J. Immun.* **125**, 118-128 (1980).
11. Yowell, R. L., Araneo, B. A., Miller, A. & Sercarz, E. E. *Nature* **279**, 70-71 (1979).
12. Ikezawa, Z. *et al. J. Immun.* **131**, 1646-1649 (1983).
13. Oki, A. N. & Sercarz, E. E. *J. exp. Med.* **161**, 897-911 (1985).
14. Waters, R. H., Pilarski, L. M., Wegmann, T. G. & Diener, E. *J. exp. Med.* **149**, 1134-1151 (1979).

Molecular cloning and expression of cDNA for human granulocyte colony-stimulating factor

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Granulocyte colony-stimulating factor (G-CSF) is a member of the CSF family of hormone-like glycoproteins that regulate haematopoietic cell proliferation and differentiation^{1,2}, and G-CSF almost exclusively stimulates the colony formation of granulocytes from committed precursor cells in semi-solid agar culture². Recently, Nomura *et al.*³ have established a human squamous carcinoma cell line (designated CHU-2) from a human oral cavity tumour which produces large quantities of CSF constitutively, and the CSF produced by CHU-2 cells has been purified to homogeneity from the conditioned medium. We have now determined the partial amino-acid sequence of the purified G-CSF protein, and by using oligonucleotides as probes, have isolated several clones containing G-CSF complementary DNA from the cDNA library prepared with messenger RNA from CHU-2 cells. The complete nucleotide sequences of two of these cDNAs were determined and the expression of the cDNA in monkey COS cells gave rise to a protein showing authentic G-CSF activity. Furthermore, Southern hybridization analysis of DNA from normal leukocytes and CHU-2 cells suggests that the human genome contains only one gene for G-CSF and that some rearrangement has occurred within one of the alleles of the G-CSF gene in CHU-2 cells.

Human G-CSF was purified to homogeneity from the conditioned medium of the human squamous carcinoma cell line CHU-2 as described elsewhere³, then the NH₂-terminal amino-acid sequences of several peptides obtained by tryptic digestion and two major peptides generated by chemical cleavage with cyanogen bromide were determined. One of the tryptic fragments showed an amino-acid sequence of Ile-Trp-Gln-Met-Glu-Glu-Leu-Gly-Met, and a 30-nucleotide oligonucleotide (IWQ probe, ATITGGCA^ΔCA^ΔATGGA^ΔGA^ΔCTIGGIATG) was synthesized chemically. This oligonucleotide contained deoxyinosine at three positions⁴ where three or four different nucleotides can be assigned due to codon degeneracy. G-CSF mRNA was prepared from the human CHU-2 cell line and two different cDNA libraries were constructed using either pBR322 as vector (library A) or the λgt10 vector⁵ system (library B). The IWQ probe was labelled with ³²P, and library A was

Table 1 G-CSF activity in the culture medium of COS cells transfected with plasmid pHGA410

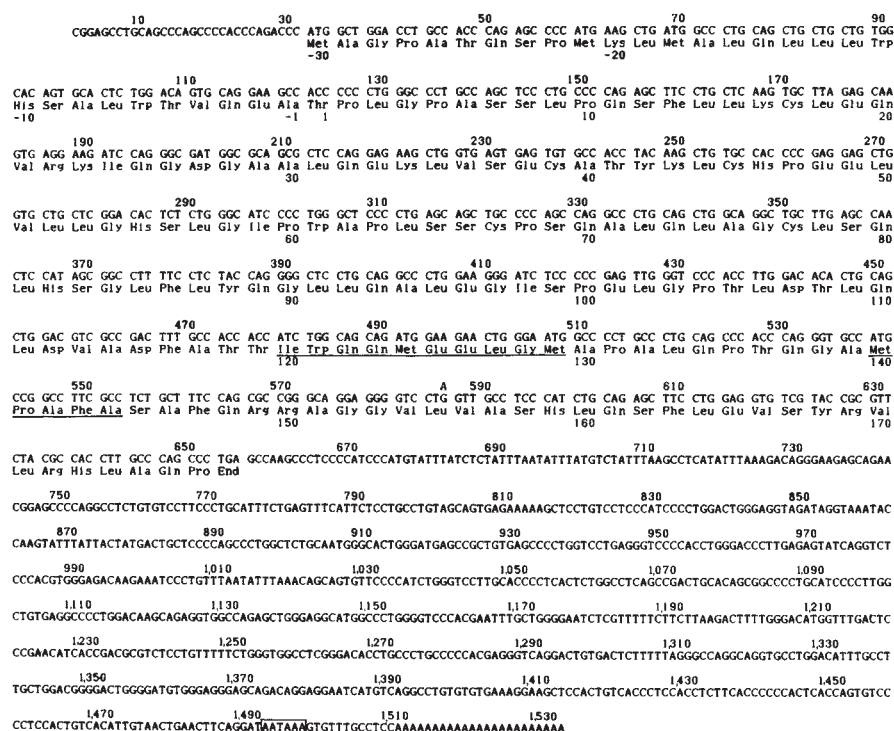
Transfected with		Human bone marrow			Mouse bone marrow	
		(no. of colonies)				
pdKCR	(a)	0	0	0	0	0
	(b)	0	0	0	NT	NT
pHGA410	(a)	104	128	128	51	42
	(b)	54	48	51	NT	NT
Purified G-CSF		72	50	97	47	53
Saline		0	0	0	0	0

COS cells (2×10^6) were seeded on a dish of 9 cm diameter with 10 ml of Dulbecco's minimal essential medium (Nissui) containing 10% fetal calf serum (Gibco). The cells were transfected with 20 µg of pHGA410 or pdKCR DNA using DEAE-dextran (Pharmacia) essentially as described elsewhere¹⁰, and treated with 1 mM chloroquine (Sigma) for 2 h. After incubation for 72 h, the medium (40 ml) was collected and G-CSF was partially purified by various procedures, including reverse-phase HPLC essentially as described by Nomura *et al.*³. The eluate from the HPLC column was lyophilized and dissolved in 2 ml of Iscove's modified medium containing 10% fetal calf serum and assayed for CSF activity as described previously²⁵. Briefly, for the human cell assay, 2×10^5 non-adherent bone marrow cells obtained from normal volunteers were added to 35-mm Petri dishes in a total volume of 1 ml of McCoy's 5A medium containing 15% fetal calf serum, 0.3% agar and 0.1 or 0.05 ml of the test sample. Cultures were incubated for 7 days at 37 °C, and colonies containing more than 50 cells were counted. For the mouse cell assay, 5×10^4 cells from a C3H/He mouse were incubated in 1 ml of McCoy's 5A medium containing 40% horse serum, 0.7% agar and 0.1 ml of the test sample. After incubation at 37 °C for 5 days, colonies consisting of more than 50 cells were counted. The assays were carried out in triplicate (for human cells) or duplicate (for mouse cells), and for the assay with human cells, 100 µl (a) or 50 µl (b) of CSF was tested. As a control, a homogeneous preparation of human G-CSF (20 ng) or saline was added to the incubation mixture instead of the test sample. NT, not tested.

screened. One clone gave a positive result and was designated pHCS-1. This clone contained a 308-base-pair (bp) insert whose nucleotide sequence confirmed the presence of a homologous region with our probe and whose amino-acid sequence, deduced from the nucleotide sequence, was identical to that of homogeneous human G-CSF. The nucleotide sequence of pHCS-1 also revealed that it can code for 83 carboxy-terminal amino acids of G-CSF, which is about half the number of amino acids expected for G-CSF. Using the cDNA insert of pHCS-1 as probe, five clones were then isolated from cDNA library B and designated as λgt-1 to λgt-5. *Eco*RI restriction enzyme digestion of DNAs from these clones released inserts of 3.5, 1.3, 0.75, 1.6 and 1.5 kilobases (kb), respectively, and these were subcloned into the *Eco*RI site of pBR327 (denoted as pBRG-1 to pBRG-5). As 1.5 kb of cDNA is long enough to encode the G-CSF protein (relative molecular mass (M_r) 19,000)³, two clones, pBRG-4 (~1.6 kb insert) and pBRG-5 (~1.5 kb insert), were further characterized by restriction enzyme analysis and nucleotide sequence determination.

Figure 1 shows the complete nucleotide sequence of the pBRG-4 insert (1,508 nucleotides long excluding the poly(A) tract). Apart from the fact that pBRG-5 insert is 8 nucleotides shorter at its 5' terminus than the pBRG-4 insert, and the poly(A) tract of pBRG-4 at the 3' terminus is about 80 bases long while that of pBRG-5 is 17 bases long, the nucleotide sequences of pBRG-5 and pBRG-4 are identical. Furthermore, comparison of the nucleotide sequences of pBRG-4 and pHCS-1 revealed a single difference at nucleotide position 586 (the third base in the Leu 155 codon), which does not change the amino-acid sequence. The cDNA in the pBRG-4 clone appeared to be of full length, as Northern hybridization analysis of CHU-2 mRNA with the cDNA of pBRG-4 revealed a band of ~1,600 nucleo-

To prove that the cloned cDNA of pBRG-4 actually encodes human G-CSF, used a mammalian cell system to express the cDNA. The 720-bp *EcoRI*/*Aha*III fragment containing the G-CSF coding sequence (Fig. 1) was recloned into the expression vector pdkCR (refs 17, 18), and the resultant plasmid (pHGA410), which contains the G-CSF cDNA in the same orientation relative to the simian virus 40 early promoter, was used to transfect COS cells¹⁹. After 72 h, the G-CSF secreted into the medium was assayed for CSF activity, either directly or after partial purification, using human or mouse bone marrow cells. As a control, the vector plasmid pdkCR, containing no cDNA insert, was transfected into COS cells and the COS cell supernatant was processed as described above. When the medium from pHGA410-transfected COS cells was assayed directly for CSF, no activity was detected. In contrast, the partially purified sample from the pHGA410-transfected COS cell supernatant showed CSF activity in both human and mouse bone marrow cells (Table 1). No CSF activity was detected in the sample purified from the medium of COS cells transfected with the pdkCR vector. To confirm that the colonies formed by the pHGA410-transfected COS cell supernatant are



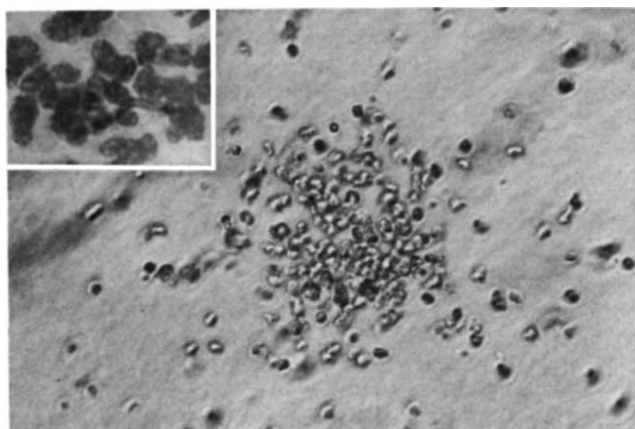


Fig. 2 Phase microscopic appearance of a human bone marrow colony stimulated by a cDNA-directed human G-CSF preparation. Inset, cells stained with Wright-Giemsa. Methods for the transfection of COS cells, purification of G-CSF from the medium conditioned with COS cells and assay for G-CSF using human bone marrow cells were as described in Table 1 legend. On day 7, several colonies were picked up on a glass plate and stained with Wright-Giemsa.

granulocyte colonies, the colonies from the human bone marrow CSF assay were examined by Wright-Giemsa and double esterase staining (Fig. 2). In most colonies, all the cells were positive for specific esterase, showing segmented or stubbed nuclei, and no colonies consisting of eosinophilic granulocytes were observed. These properties are similar to those of human CSF- β partially purified from medium conditioned with human placenta²⁰ or 5637 bladder carcinoma cells²¹. The failure to detect CSF activity directly in the transfected COS cell supernatant is probably due to a low specific activity of protein or a low expression of the protein in COS cells. In this regard, we have recently found²² a second G-CSF cDNA which has a three-amino-acid deletion at around amino acid 35 compared with the cDNA described in Fig. 1. When this cDNA was expressed in COS cells, CSF activity was detected in the COS cell supernatant without purification.

Several tumours²³⁻²⁵ are known to produce haematopoietic regulators which function within the granulocyte-macrophage pathway. The specific expression of G-CSF by these cells was investigated by preparing mRNAs from human lung carcinoma (OTUK)²⁵, human lower jaw carcinoma (LJC-1-JCK)²⁴ and CHU-2 cells, which produce proteins possessing CSF activity, and the human B-cell line Namalwa which does not produce CSF. Figure 3a shows the Northern hybridization analysis of the mRNAs using G-CSF cDNA as probe. About 1.6 kb of mRNA hybridized very strongly with mRNA from CHU-2 cells and weakly with mRNA from LJC-1-JCK cells, while no signal was detected with mRNA from OTUK and Namalwa cells. These results indicate that CHU-2 and LJC-1-JCK cells actually synthesize G-CSF mRNA. Furthermore, proteins showing CSF activity which are produced by OTUK cells are probably not G-CSF, but rather for example, GM-CSF²⁶, pluripotent-CSF²⁷ or haemopoietin-1²⁸, which act within the granulocyte-macrophage pathway.

To determine how many G-CSF genes exist in the human genome, and whether any amplification or rearrangement of the gene has occurred in CHU-2 cells producing G-CSF constitutively, DNAs from human normal leukocytes and CHU-2 cell were analysed by Southern hybridization. In DNA from leukocytes, a single DNA fragment was detected after digestion with any of four restriction enzymes used (Fig. 3b), which suggests that human G-CSF is encoded by a single gene; this has been confirmed by molecular cloning of the chromosomal gene for human G-CSF²². On the other hand, in DNA from

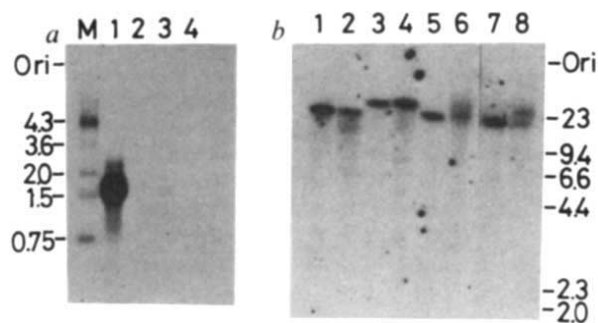


Fig. 3 Characterization of the mRNA and genomic gene coding for human G-CSF. *a*, Northern hybridization analysis of G-CSF mRNA. The following cells were used to prepare poly(A) RNA: lane 1, human squamous carcinoma, CHU-2 cells; lane 2, human lung carcinoma, OTUK cells; lane 3, human lower jaw carcinoma, LJC-1-JCK; lane 4, human B-cell line, Namalwa; lane M, size marker, a mixture of 5'-³²P-labelled *Eco*RI, *Eco*RI/*Pst*I and *Eco*RI/*Pvu*II-digested pBR322. The numbers are the sizes of the marker DNAs (in kb). *b*, Southern hybridization of the G-CSF gene. Sources of DNA are: lanes 1, 3, 5, 7, human normal leukocytes; lanes 2, 4, 6, 8, CHU-2 cells. Restriction enzymes used are: lanes 1, 2, *Eco*RI; lanes 3, 4, *Bgl*II; lanes 5, 6, *Hind*III; lanes 7, 8, *Bam*HI.

Methods. *a*, Poly(A) RNA was prepared from various cell lines³⁴. Poly(A) RNAs (5 μ g) were electrophoresed through 1.2% agarose gel containing formaldehyde³⁴ and blotted onto a nitrocellulose filter. The ³²P-labelled *Eco*RI cDNA insert of pBRG-4 was used for hybridization in stringent conditions³⁶. *b*, High-*M_r* DNAs were prepared from human leukocytes, or CHU-2 cells as described previously³⁴. DNAs (10 μ g) were digested with various restriction enzymes, electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose filter. The filter was hybridized with the ³²P-labelled *Eco*RI cDNA insert of pBRG-4 as described above.

CHU-2 cells, additional DNA fragments were observed after digestion with *Eco*RI (Fig. 3b, lane 2), *Hind*III (lane 6) or *Bam*HI (lane 8), which may indicate that some rearrangement but no amplification of the G-CSF gene had occurred in CHU-2 cells. Therefore, it is possible that alterations within one of the alleles of the G-CSF gene or its flanking regions led to the constitutive production of G-CSF in CHU-2 cells. Detailed analysis of the G-CSF chromosomal gene structure in normal and CHU-2 cells may reveal the mechanism of the constitutive synthesis of G-CSF in CHU-2 cells. The availability of human G-CSF cDNA should facilitate the production of large quantities of human G-CSF in appropriate host cells and study of the role of this factor in haematopoietic cell proliferation and differentiation.

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- Burgess, A. W. & Metcalf, D. *Blood* **56**, 947-958 (1980).
- Metcalf, D. *Science* **229**, 16-22 (1985).
- Nomura, H. *et al.* *EMBO J.* (submitted).
- Takahashi, Y. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **82**, 1931-1935 (1985).
- Huynh, T., Young, R. A. & Davis, R. W. in *DNA Cloning Techniques, A Practical Approach* (ed. Glover, D. M.) 49-78 (IRL Press, Oxford, 1985).
- Kozak, M. *Nucleic Acids Res.* **12**, 857-872 (1984).
- Kozak, M. *Cell* **15**, 1109-1123 (1978).
- Winkler, R. J. in *Hormonal Proteins and Peptides* (ed. Li, C. H.) 1-15 (Academic, New York, 1973).
- Takahashi, N., Takahashi, Y. & Putnam, F. W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 2021-2025 (1984).
- Lee, F. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **82**, 4360-4364 (1985).
- Wong, G. G. *et al.* *Science* **228**, 810-815 (1985).
- Yokota, T. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **81**, 1070-1074 (1984).
- Fung, M. C. *et al.* *Nature* **307**, 233-237 (1984).
- Nicola, N. A. & Metcalf, D. *Proc. natn. Acad. Sci. U.S.A.* **81**, 3765-3769 (1984).
- Wilbur, W. J. & Lipman, D. J. *Proc. natn. Acad. Sci. U.S.A.* **80**, 726-730 (1983).
- Kanehisa, M. *Nucleic Acids Res.* **10**, 183-196 (1982).

17. O'Hare, K., Benoist, C. & Breathnach, R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 1527-1531 (1981).
18. Fukunaga, R., Sokawa, Y. & Nagata, S. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5086-5090 (1984).
19. Gluzman, Y. *Cell* **23**, 175-182 (1981).
20. Nicola, N. A., Metcalf, D., Johnson, G. R. & Burgess, A. W. *Blood* **54**, 614-627 (1979).
21. Nicola, N. A., Begley, C. G. & Metcalf, D. *Nature* **314**, 625-628 (1985).
22. Nagata, S. *et al. EMBO J.* (submitted).
23. Asano, S. *et al. Br. J. Cancer* **41**, 689-694 (1980).
24. Sato, N. *et al. Cancer* **43**, 605-610 (1979).
25. Asano, S. *et al. Blood* **49**, 845-852 (1977).
26. Gasson, J. C. *et al. Science* **226**, 1339-1342 (1984).
27. Welte, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 1526-1530 (1985).
28. Jubinsky, P. T. & Stanley, E. R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 2764-2768 (1985).
29. Walseth, T. F. & Johnson, R. A. *Biochim. biophys. Acta* **562**, 11-31 (1979).
30. Land, H., Grez, N., Hansen, H., Lindermaier, W. & Schuetz, G. *Nucleic Acids Res.* **9**, 2251-2266 (1981).
31. Gubler, U. & Hoffman, B. J. *Gene* **25**, 263-269 (1983).
32. Taub, F. & Thompson, E. B. *Analyt. Biochem.* **126**, 222-230 (1982).
33. Hall, A. & Brown, R. *Nucleic Acids Res.* **13**, 5255-5268 (1985).
34. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
35. Benton, W. D. & Davis, R. W. *Science* **196**, 180-182 (1977).
36. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683-3687 (1979).
37. Messing, J. *Meth. Enzym.* **101**, 20-78 (1983).

'Brain-specific' transcription and evolution of the identifier sequence

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A recent model for the transcriptional control of gene expression in neural cells involves a dispersed repetitive DNA sequence termed the identifier (ID) sequence¹. However, the model is based on circumstantial evidence from studies on rat brain gene expression²⁻⁴. Furthermore, available data are complicated by observations from several laboratories which suggest that the ID sequence is a family of mobile genetic elements⁵⁻¹⁰. Although this does not preclude a role for some family members in regulating gene expression, the contention that these sequences are transcribed tissue-specifically (refs 1-4, but see ref. 11) is not proof of such a role¹². We have now measured the genomic copy number and tissue pattern of transcription of ID sequences in the rat, mouse and hamster, and have found that ID-homologous, BC1-like² RNAs are restricted to brain in all three species, but that ID-homologous transcripts occur in total cellular RNAs of brain, liver and kidney of all three organisms. The genomic copy number of the ID sequences varies over two orders of magnitude between these species. Our data suggest that most ID sequences in these genomes are dispersed at random with respect to transcription units. A *cis*-acting, transcriptional-level controlling role for the ID therefore seems unlikely.

The function of the ID sequence cannot be tested directly, but because the idea of multiple independent origins for the vertebrate brain seems improbable, a functional role for the ID sequence requires that at least two evolutionary criteria be fulfilled: (1) a homologous dispersed repetitive-sequence family which is transcribed neurone-specifically should be present in the genomes of related organisms and (2) that sequence family should contain enough members to be present in most neurone-specific genes. Our experiments assessed ID sequences for these characteristics.

We isolated total cellular RNA from the brain, liver and kidney of rat, mouse and hamster by guanidinium isothiocyanate/hot phenol extraction¹³. These RNAs were fractionated on a denaturing polyacrylamide gel, electroblotted and probed with the rat ID clone p2A120 (ref. 2) (Fig. 1). Both BC1 and BC2 RNAs were detected in the rat brain sample, as well as a previously unreported salt-soluble species of ~70 nucleotides. (Previous studies indicate that a substantial fraction of brain messenger RNAs are not polyadenylated¹⁴⁻¹⁶. These RNAs, as well as BC2 RNA¹, would be eliminated by oligo(dT)-cellulose

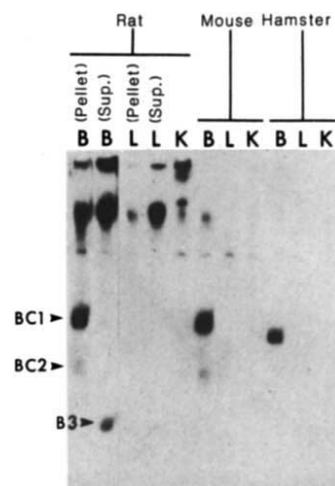


Fig. 1 Northern blot analysis of ID-homologous RNAs extracted from brain (B) liver (L) and kidney (K) of rat, mouse and hamster. Total cellular RNAs were isolated by guanidinium isothiocyanate/hot phenol extraction¹³. In some cases RNAs were fractionated by precipitation in 2.5 M NaCl at -8 °C for 6-10 h. Total cellular or salt precipitated RNAs (20 µg) were denatured at 67 °C in 30% dimethyl sulfoxide⁴ and subjected to electrophoresis on denaturing 6% polyacrylamide gels¹³. The RNAs were then transferred to Zetabind nylon membrane (AMF Cuno, Meriden, Connecticut) by electroblotting¹³. The electrophoretically purified 580-bp *Pst*I fragment of p2A120² was labelled *in vitro* with [α -³²P]dCTP (3,000 Ci mmol⁻¹, Amersham) to specific activities of 5×10^7 - 2×10^8 c.p.m. µg⁻¹ by nick translation¹³. Hybridization was done at 42 °C in 50% formamide/5×SET (0.75 M NaCl/0.15 M Tris-HCl pH 8/10 mM EDTA)/1×Denhardt's/50 µg ml⁻¹ salmon-sperm DNA/0.5% SDS for 36-40 h. Four stringency washes (40 min each) were done in 2×SSC/0.1% SDS at 60-62 °C. Blots were then exposed to Kodak XAR-5 film with Dupont Lightning Plus intensifying screens at -80 °C for 15-48 h. The higher *M_r* smears hybridized in the salt supernatant fractions are probably due to DNA contamination, as samples in this figure were not treated with DNase I. Rats were Sprague-Dawley strain, mice were strain BALB/c ByJ and hamsters were Golden Syrian.

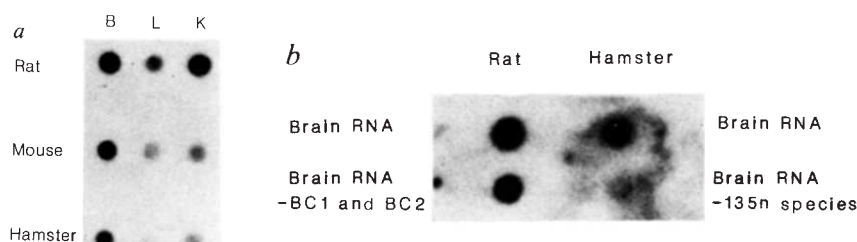
chromatography. We fractionated BC1 and BC2 into the same compartment and enriched for larger RNA species by salt precipitation.) ID-homologous RNAs of similar mobility to BC1 and BC2 were found in total mouse brain RNA and a 135-nucleotide species was found in total hamster brain RNA. The level of hybridization to larger species (the smear which runs at the limiting mobility of the gel, presumed to be heterogeneous nuclear RNA^{1,11}) was much greater in the rat than in the mouse or hamster. Under the hybridization and washing conditions used in Fig. 1, another RNA species that co-migrated with 7SL RNA was detected after prolonged autoradiographic exposure in all three tissues of all three organisms. We do not know whether this RNA is in fact 7SL RNA, but weak homology between *Alu* sequences and the ID sequence does exist (22 of the first 30 nucleotides of CHO *Alu* clone 49 (ref. 17) are homologous with the ID clone p2A120, and similar levels of homology occur with many published *Alu* sequences).

Having shown that the nucleotide sequence of the ID is at least partially conserved between the rat, mouse and hamster, we used dot-blot hybridization to examine the relative level of ID-homologous transcripts present in salt-precipitated RNA from brain, liver and kidney of each organism. Figure 2a shows that ID-homologous transcripts are present in liver and kidney samples of all three organisms. Densitometric tracing of lighter exposures of Fig. 2a indicated that there were approximately four and eight times as many ID-homologous transcripts in rat brain as in kidney and liver, respectively. To determine what fraction of ID-homologous brain RNA was caused by the presence of BC1 and BC2 RNAs in rat and the 135-nucleotide RNA

Fig. 2 *a*, Dot-blot hybridization assaying levels of ID-homologous transcripts in brain (B), liver (L) or kidney (K) RNAs from rat, mouse and hamster. *b*, ID-homologous transcripts of rat and hamster brain from which the BC1 and BC2 RNAs and the 135-nucleotide RNA respectively have been removed.

Methods. *a*, Salt-precipitated RNA samples were first treated with DNase I (50 $\mu\text{g ml}^{-1}$, Boehringer Mannheim) in the presence of 2,000 U ml^{-1} RNasin (Promega Biotec) for 30 min at 37 °C.

After phenol/chloroform extraction and ethanol precipitation, the RNA was redissolved and the concentration estimated spectrophotometrically¹³. There was no apparent quantitative or qualitative difference in the ethidium bromide staining pattern of RNAs treated in this manner and run on denaturing 6% polyacrylamide gels, as compared with untreated samples (data not shown). All of the small RNAs normally present were clearly visible⁴. 8 μg of each RNA was denatured in 50% formamide at 75 °C for 10 min, cooled in ice H_2O , made 1 M in NH_4OAc and filtered onto a nylon membrane using a Hybri-Dot manifold (Bethesda Research Laboratories). The absence of contaminating DNA was confirmed by the complete lack of hybridization to NaOH-treated control samples in adjacent spots. Hybridization and washing were done as in Fig. 1 with *in vitro* labelled 580-bp *Pst*I fragment from p2A120. Probe was judged to be in effective excess by the linearity of autoradiographic response over 1, 2 and 4 μg of rat brain RNA treated as above and present on the same blot (data not shown). *b*, Salt-precipitated rat brain or hamster brain RNA was fractionated on denaturing 5% polyacrylamide gels. The RNA was extracted from the gel¹³ either excluding or including regions of the gel containing BC1 and BC2 RNA in the case of rat or the 135-nucleotide species in the case of hamster. To ensure effective extraction of larger species, regions of the gel containing RNAs larger than 7SL RNA were treated with 10 mM NaOH for 15 min at room temperature. The final products of this extraction procedure ran as a smear between 80 and 500 nucleotides in length on denaturing 6% polyacrylamide gels; 5 μg of each RNA was treated as in *a*.



in hamster, we removed these species from samples of rat and hamster brain RNA by fractionation on a denaturing 5% polyacrylamide gel, followed by extraction of the RNA from the gel, either excluding or including species of these relative molecular masses (M_s). Densitometric tracing of Fig. 2*b* indicates that >90% of the ID-homologous transcripts present in hamster brain RNA are caused by the 135-nucleotide species, whereas approximately half of ID-homologous rat brain RNAs result from BC1 and BC2 RNAs.

Using this estimate, we conclude that ID-homologous transcripts (other than BC1 and BC2) are two- to fourfold more prevalent in rat brain RNA than in liver or kidney RNA. Our results for the higher level of ID-homologous transcripts present in the rat brain agree qualitatively with those of Sutcliffe and co-workers, but the rather small quantitative differences and the fact that we observe similar differences between liver and kidney lead us to believe that these differences are artefactual¹¹.

We also measured the copy number of ID-homologous sequences in the genomes of rat, mouse, hamster, guinea pig and rabbit by several different methods. Figure 3*a* shows that, at the same hybridization stringency used to detect ID-homologous transcripts in Figs 1 and 2, the rat genome contains at least four times as many copies of the ID sequence as do the genomes of any of the other organisms. Prolonged exposure of this autoradiogram revealed a faint signal in the 50-ng mouse DNA spot which is $\sim 1/10$ th as intense as the rat 50-ng spot.

Figure 3*b* shows an identical dot blot hybridized with a 900-base-pair (bp) *Sau*3A fragment containing the rat ID-homologous portion of a positive mouse library phage clone (designated pM3A-2, and which gives a pattern of hybridization on Northern blots identical to that obtained with p2A120; data not shown). The result is qualitatively the same; there are an order of magnitude more mouse-ID-homologous sequences in the rat genome than in the mouse, hamster, guinea pig or rabbit genomes.

Of 10^4 plaques of the Sargent and Bonner rat genomic library¹⁸ screened with p2A120, 39% were positive and thus contained at least one copy of the ID sequence (Table 1), implying that there are $\sim 135,000$ copies per genome. This agrees well with the estimate of Sutcliffe *et al.* that 43% of randomly chosen rat clones contain at least one copy of the ID sequence³. Only 3.6% of 10^3 plaques of a mouse genomic library hybridized to either p2A120 or the mouse ID clone pM3A-2, implying $\sim 12,000$ copies of the ID sequence per mouse genome. We constructed a partial genomic library of hamster DNA *Sau*3A fragments in the plasmid vector pEMBL8⁺ and screened 1,800 clones containing inserts averaging 600 bp in length (a significant fraction of

recombinant plasmids contained more than one insert) with both the rat and mouse ID inserts of p2A120 and pM3A-2. We detected no homologous sequences with either clone, indicating a maximum of 4,700 copies of the ID per hamster genome. Comparable estimates were obtained by saturation hybridization with either p2A120 or pM3A-2 as driver (Table 1). Between 11 and 20% of the counts hybridized were released by treatment with S_1 nuclease, providing a rough estimate of between-member divergence of ID sequences present in each genome.

With regard to the model for control of gene expression proposed by Sutcliffe and co-workers¹, Owens *et al.*¹¹ have recently demonstrated that there are no differences in the level of ID-homologous transcripts in brain, liver or kidney hnRNA in either the rat or mouse. Their estimate of the relative difference in absolute amount of ID-homologous hnRNA between rat and mouse¹¹ is in good agreement with the difference we measure in ID copy number between these species. These observations

Table 1 Identifier sequence copy number

	Saturation hybridization p2A120	pM3A-2	Library screen
Rat	133,000 (13)	120,000 (ND)	135,000 (39% of 10^4 plaques)
Mouse	19,500 (19)	15,800 (21)	12,500 (3.6% of 10^3 plaques)
Hamster	1,600 (20)	2,500 (11)	<4,700 (0 of 1,000 kb)

Saturation hybridizations were done with rat ID clone p2A120 or mouse ID clone pM3A-2 as driver. Samples of plasmid DNA were linearized by cleavage with *Eco*RI, denatured and fixed to 2.4-cm Zetabind[®] circles. Samples of each genomic DNA were cleaved with *Sau*3A (Boehringer Mannheim) to generate fragments averaging 300 bp in length (minimizing 'network' formation) and *in vitro* labelled with [α - ^{32}P]dCTP and [α - ^{32}P]dATP to specific activities of 9.4×10^7 – 1.2×10^8 c.p.m. μg^{-1} . Hybridizations were carried out for 39 h at 42 °C in 50% formamide/5 $\times$ SSC/20 mM Na-phosphate pH 6.7/1 $\times$ Denhardt's/50 μg salmon sperm DNA ml^{-1} and four stringency washes done at 60–62 °C in 2 $\times$ SSC/0.1% SDS. Filter-bound DNA was judged to be in excess because 1, 3.3 and 10 μg of each plasmid DNA bound the same fraction of input c.p.m. Filters containing 3.3 μg of plasmid DNA were then incubated with increasing amounts of labelled genomic DNA. The fraction of input c.p.m. bound was linear over the range tested. The c.p.m. bound were corrected for the efficiency of hybridization (as measured by hybridization of labelled p2A120 or pM3A-2 to homologous filter-bound DNA, 39% in the case of p2A120, 35% in the case of pM3A-2) and copy number was calculated as (corrected c.p.m. bound/input c.p.m.) $\times 2.5 \times 10^9$ bp (rodent genomic complexity) $\div$ 82 bases (length of the ID sequence). Numbers in parentheses in the saturation hybridization columns represent the fraction of c.p.m. bound released by S_1 nuclease treatment (ND, not determined). Phage and plasmid library screening were done as described elsewhere¹³ and copy number calculated also as described previously⁷. The mouse genomic library was obtained from Y. Nishioka and constructed in the laboratory of S. Tonegawa by ligating partial *Eco*RI digests of mouse genomic DNA to λ Charon 4a arms.

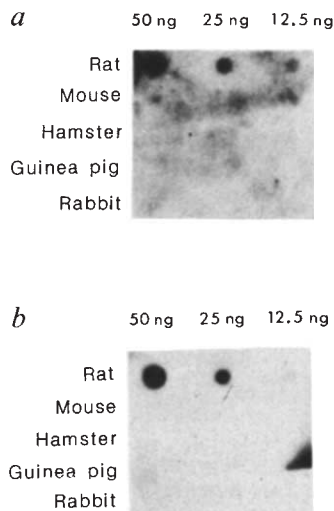


Fig. 3 Estimation of the relative copy number of ID-homologous sequences in the genomes of rat, mouse, hamster, guinea pig and rabbit. The hybridization probes are the *in vitro* labelled 580-bp *Pst*I fragment of p2A120 (a) and the *in vitro* labelled 900-bp *Sau*3A fragment from the mouse ID clone pM3A-2 (see test) (b). DNA prepared from the liver of each organism²⁰ was digested with *Eco*RI (Boehringer Mannheim) and fixed to a nylon membrane as described elsewhere²¹.

suggest that the vast majority of ID sequences within these genomes are dispersed approximately at random with respect to transcription units. These data further suggest that the demonstrated brain specificity of 160- or 135-nucleotide BC1-like RNAs is not caused by the transcription of thousands of different 82-nucleotide IDs located in brain gene introns¹, but is more probably the result of transcription of a small subset of ID-homologous genes that specifically code for this RNA. Unequivocal data on the sequence diversity of BC1 RNA will help to determine whether the between-species differences in ID copy number reflect the generation of pseudogenes or transposition processes like those invoked for the derivation of *Alu* sequences from 7SL RNA¹⁹.

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1. Sutcliffe, J. G., Milner, R. J., Gottesfeld, J. M. & Reynolds, W. *Science* **225**, 1308-1315 (1984).
2. Sutcliffe, J. G., Miller, R. J., Bloom, F. E. & Lerner, R. A. *Proc. natn. Acad. Sci. U.S.A.* **79**, 4942-4946 (1982).
3. Milner, R. J., Bloom, F. E., Lai, C., Lerner, R. A. & Sutcliffe, J. G. *Proc. natn. Acad. Sci. U.S.A.* **81**, 713-717 (1984).
4. Sutcliffe, J. G., Milner, R. J., Gottesfeld, J. M. & Lerner, R. A. *Nature* **308**, 237-241 (1984).
5. Barta, A., Richards, R. I., Baxter, J. D. & Shine, J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4867-4871 (1981).
6. Schuler, R. A., Weber, J. L. & Gorski, J. *Nature* **305**, 159-160 (1983).
7. Lemischka, I. & Sharp, P. A. *Nature* **300**, 330-335 (1982).
8. Watanabe-Nagasu, N. *et al. Nucleic Acids Res.* **11**, 1791-1801 (1983).
9. Hastie, N. *Trends Genet.* **1**, 37 (1985).
10. Page, G. L., Smith, S. & Goodman, H. M. *Nucleic Acids Res.* **9**, 2087-2103 (1981).
11. Owens, G. P., Chaudhri, N. & Hahn, W. E. *Science* **229**, 1263-1265 (1985).
12. Davidson, E. H. & Posakony, J. W. *Nature* **297**, 633-635 (1982).
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
14. Chikaraishi, D., *Biochemistry* **18**, 3249-3258 (1979).
15. Van Ness, J., Maxwell, J. H. & Hahn, W. E. *Cell* **18**, 1341-1349 (1979).
16. Hahn, W. E., Chaudhri, N., Beck, L., Wilber, K. & Peffley, D. *Cold Spring Harb. Symp. quant. Biol.* **48**, 465-475 (1983).
17. Haynes, J. R. & Jelinek, W. R. *Proc. natn. Acad. Sci. U.S.A.* **78**, 6130-6134 (1981).
18. Sargeant, T. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 3256-3260 (1979).
19. Ullu, E. & Tschudi, C. *Nature* **312**, 171-172 (1984).
20. Scott, M. P. *et al. Cell* **35**, 763-776 (1983).
21. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. *Nucleic Acids Res.* **7**, 1541-1552 (1979).

Rearrangement of two distinct T-cell γ -chain variable-region genes in human DNA

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Selective cloning procedures for T-cell-specific complementary DNAs have revealed the existence of a gene designated γ as well as the main antigen receptor α - and β -chain genes²⁻¹⁰. The γ -chain genes undergo rearrangement during T-cell differentiation but the patterns and complexity of such rearrangements differ markedly in mouse¹¹⁻¹³ and human¹⁴. In mouse, a panel of cytotoxic T-lymphocyte clones exhibit the same rearrangement pattern with a γ -chain gene probe¹¹ and a set of three γ -chain variable (*V*) genes have been identified in the DNA¹². Clonal diversity in mouse seems to be confined to *V*-*J* (joining) regions¹¹. In contrast, human T-cell lines exhibit diverse rearrangements¹⁴ suggestive of a family of differing *V_γ* genes variously rearranging to the two γ -chain constant (*C*) region genes^{14,15}. Here we report the cloning of two very different *V_γ* genes rearranged to *J* segments upstream of the two human *C_γ* genes. Both *V_γ* genes are rearranged productively but nucleotide sequence comparison shows that they possess very little homology with each other. This shows that human T-cell *V_γ* genes exist which differ significantly from each other at the nucleotide level and that such diverse genes can be usefully rearranged in different T cells.

A further investigation of the diversity in the human T-cell γ -chain gene locus was carried out by cloning rearranged *V_γ* genes from λ phage libraries made from two different T-cell lines. We first determined the location of the *J_γ* segments. A probe from upstream of the *C_γ1* gene was previously used to detect rearrangement in various human T cells¹⁴. All such rearrangements were observed in a 2-kilobase (kb) *Hind*III fragment found next to each of the *C_γ* genes; therefore it seems likely that they include the *J_γ* segments. The nucleotide sequences of the 2.1-kb *Hind*III fragments from upstream of *C_γ1* and *C_γ2* were therefore determined. Each sequence was scanned for



Fig. 1 Nucleotide sequence comparison of human *J_γ1* and *J_γ2* regions. Nucleotide sequences are shown extending from *Hind*III to *Eco*RI sites of M13H60 (ref. 14) and the equivalent fragment of the γ 2-chain gene, determined and aligned using random sequencing methods¹⁸⁻²⁰. The relevant restriction sites are indicated at the start and end of the sequence and nucleotide differences are starred. The *J_γ* sequences are shown together with the conserved nanomer (AGTTTGA) and heptamer sequences (CACTGTG) (lines above and below); the RNA splice donor sites are arrowed.

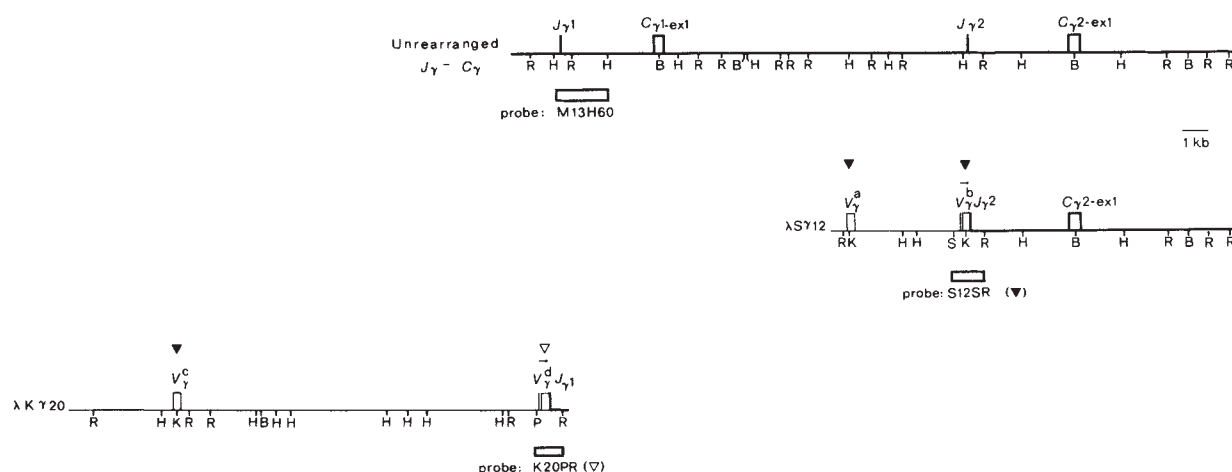


Fig. 2 Restriction maps of germline and rearranged γ -chain genes. λ phage clones were isolated from either a genomic library prepared from SUPT1 cells¹⁶ (λ S γ 12) or from Karpas 1010 (λ K γ 20). Both libraries were prepared in λ 2001 (ref. 21). Top line, unrearranged gene organization of J_γ - C_γ region^{14,15} with only the first exon of C_γ genes indicated. Middle line, restriction map of λ clone S γ 12; bottom line, restriction map of the γ clone K γ 20. Horizontal arrows, known transcriptional orientations; ∇ , V_γ genes that are cross-hybridizing species. Restriction sites indicated are: R, *EcoRI*; H, *HindIII*; B, *BamHI*; S, *SacI*; K, *KpnI* and P, *PstI* (not all S, K and P sites are given).

the presence of potential J segments and only one J segment was found in each case. The aligned nucleotide sequences, including the $J_\gamma 1$ and $J_\gamma 2$ segments, are shown in Fig. 1 from the upstream *HindIII* site to an *EcoRI* site 710 base pairs (bp) downstream. Conserved nanomer-heptamer sequences, implicated in the V - J joining process, occur immediately upstream of each J segment; the spacing of these nanomer-heptamer sequences is 12 bp. The protein coding sequences of the two J_γ segments are identical. In this respect the human and mouse γ -chain genes are similar because in mouse each C_γ gene has only one J segment¹². There is considerable sequence homology between the two regions in the human DNA, but sufficient differences exist to allow unequivocal assignment of rearranged genes to $J_\gamma 1$ or $J_\gamma 2$ (see below).

The location of the two identified human J_γ segments relative to the first exon of each C_γ gene is shown in the restriction map at the top of Fig. 2 (unrearranged J_γ - C_γ). To study the diversity of V_γ genes, we screened genomic libraries made from two different human T-cell lines (both of which display rearranged γ -chain genes¹⁴) using the M13H60 (J_γ) probe (Fig. 2, top). The restriction maps of representative clones isolated from these two libraries appear in Fig. 2. One set of clones (derived from a library of DNA from the T-cell line Karpas 1010), represented by λ K γ 20, was found by nucleotide sequencing to have a V_γ

gene segment (designated V_γ^d) joined to $J_\gamma 1$ (see below). The other set, represented by λ S γ 12, has a V_γ gene (designated V_γ^b) joined to $J_\gamma 2$ and was isolated from a phage library prepared from DNA¹⁶ of the cell line SUPT1 (ref. 17). The nucleotide sequences of the rearranged V_γ genes showed that in both cases a productive rearrangement between V_γ and J_γ had occurred (Fig. 3). In common with other V genes, both the rearranged γ -chain genes have a short leader sequence split by an intron of ~ 120 bp. Comparison of the nucleotide sequences of the two rearranged V_γ genes showed very little homology. However, some degree of homology was observed between the potential protein products of the V_γ genes (Fig. 4a). Although the precise degree of homology is difficult to assess because of high divergence, the two V_γ genes are estimated to be $\sim 30\%$ similar. Certain conserved residues are observed; for example, cysteine residues probably involved in the intra-chain disulphide bonds and the region near the J_γ segment (Tyr-Tyr-Cys-Ala). However, the two V_γ genes are clearly very different and represent two distinct V_γ families.

Two further V_γ genes were found in the human γ clones described here by cross-hybridization experiments using the probe S12RS (Fig. 2) which contains the rearranged V_γ^b gene (data not shown). One gene (designated V_γ^c ; Fig. 2) occurs ~ 14 kb upstream of the rearranged V_γ^d gene in λ K γ 20 and the

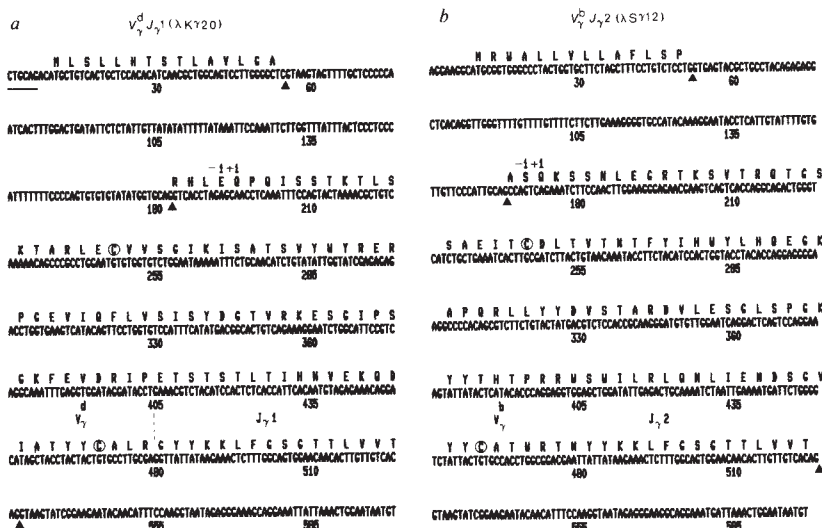


Fig. 3 Nucleotide sequences and derived protein sequences of rearranged V_γ genes. *a*, V - J junction in λ K γ 20; *b*, V - J junction in λ S γ 12.

Methods. *a*, The *PstI* site underlined at the start of the sequence was used to prepare a 1-kb *PstI*/*EcoRI* fragment from which the DNA sequence was obtained by the random procedure; only the relevant sequence region is shown. RNA splice sites are arrowed and cysteine residues involved in putative intra-chain disulphide bond are circled. The V - J junction is indicated by a vertical dotted line. *b*, The nucleotide sequence was obtained by the random cloning procedures from a 1.2-kb *SacI*/*EcoRI* fragment (identical with S12SR; Fig. 2) containing $J_\gamma 2$. Annotations and symbols are as in *a*.

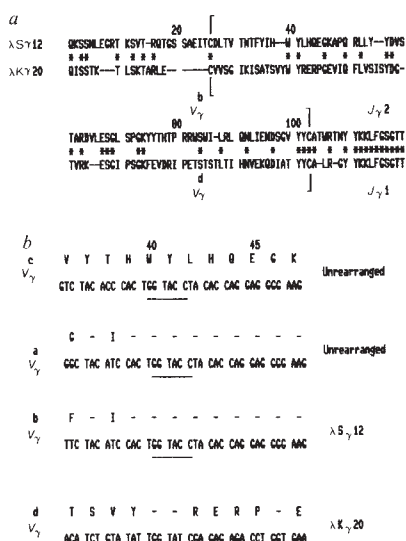


Fig. 4 Sequence comparisons of human V_γ genes. *a*, Alignment of rearranged V_γ protein sequences from λ Sy12 and λ Ky20. Sequences were aligned manually by inserting spaces as appropriate: the positions of Cys residues presumably involved in disulphide bond formation are shown. Stars represent sequence identity. *b*, Comparison of V_γ sequences around the Trp-Tyr codons 40 and 41. Dashes indicate amino-acid identity. The *Kpn*I restriction sites in V_γ^c , V_γ^a and V_γ^b are underlined. This site was used to obtain nucleotide sequence (in M13 vectors)²² of the unrearranged genes.

other gene (designated V_{γ}^b ; Fig. 2) occurs 4.5 kb upstream of V_{γ}^b in λ Sy12. Both unrearranged V genes contain a site for the restriction enzyme *Kpn*I (GGTACC). An analogous restriction site occurs in the V_{γ}^b gene of λ Sy12, part of which corresponds to the conserved codons Trp-Tyr (Fig. 4a). Confirmation that the hybridizing segments were V_{γ} genes is provided by partial nucleotide sequencing around the *Kpn*I site. These sequences are shown in Fig. 4b and compared with the corresponding regions of the V_{γ}^c and V_{γ}^d (that is, the two rearranged) genes. All the V_{γ} genes contain the conserved Trp-Tyr codons but V_{γ}^d does not retain the *Kpn*I restriction site. Figure 4b compares a short stretch of DNA sequence (around the Trp-Tyr codons) of V_{γ}^c with the other unrearranged V_{γ}^a gene and with the two rearranged genes. Both V_{γ}^a and V_{γ}^b (the latter rearranged in λ Sy12) show a high degree of similarity with V_{γ}^c but both differ from it at two positions, resulting in amino-acid replacements (these replacements are at identical positions in V_{γ}^a and V_{γ}^b). In addition the unrearranged genes V_{γ}^a , V_{γ}^c and the rearranged V_{γ}^b cross-hybridize, and therefore the data are indicative of related but non-identical V genes. On the other hand, in the region compared here, the unrearranged V_{γ}^c gene and the rearranged V_{γ}^d differ at 9 codons out of 12. Further, the probe K20PR (Fig. 2), which contains the rearranged V_{γ}^c gene segment, hybridized to the homologous segment in λ Ky20 but not the V_{γ}^c gene (data not shown), strengthening the view that the set of genes derive from two different V_{γ} families. Nucleotide sequencing shows that the V_{γ} genes are arranged in the same transcriptional orientation. This is different from the situation found in mouse DNA where two of the three identified V_{γ} genes are separated by only 2.5 kb and are arranged in a head-to-head transcriptional orientation. This is different from the situation that the two human C_{γ} genes have an associated J_{γ} segment to which V_{γ} genes can productively join. The nanomer-heptamer conserved sequences are, like the equivalent mouse γ -chain system¹², separated by 12 bp. Four human V_{γ} genes have been identified thus far and the two rearranged V_{γ} genes are rather distinct from each other at both nucleotide and protein level (Fig. 4a). This is different from the situation in mouse where limited diversity¹¹ appears to be confined to three V_{γ} genes¹², although it now seems that a further mouse V_{γ} gene exists¹³.

As far as the human γ -chain genes are concerned, our data show at least four different V_γ segments which fall into two distinct families based on the sequence homology described here. One V_γ gene family has a minimum of three members (here designated V_γ^a , V_γ^b and V_γ^c); the other family has only one member at present (designated V_γ^d). The possible existence of further members of these two families and even of further diverse human V_γ families must therefore be considered, particularly in the light of multiple rearrangements seen in human T-cell lines¹⁴. The function of the γ -chain gene product remains a matter of speculation because of the lack of data in protein expression. The apparent diversity of variable-region segments which can result from the two families of human V_γ genes identified preclude the formulation of simple models.

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1. Saito, H. *et al. Nature* **309**, 757-762 (1984).
2. Chien, Y.-H. *et al. Nature* **312**, 31-35 (1984).
3. Gascoigne, N. R. J., Chien, Y.-H., Becker, D. M., Kavalier, J. & Davis, M. M. *Nature* **310**, 387-391 (1984).
4. Hedrick, S. M., Nielsen, E. A., Kavalier, J., Cohen, D. I. & Davis, M. M. *Nature* **308**, 153-158 (1984).
5. Saito, H. *et al. Nature* **312**, 36-40 (1984).
6. Sim, G. K. *et al. Nature* **312**, 771-775 (1984).
7. Sims, J. E., Tunnacliffe, A., Smith, W. S. & Rabbitts, T. H. *Nature* **312**, 541-545 (1984).
8. Siu, G. *et al. Cell* **37**, 381-391 (1984).
9. Yanagi, Y. *et al. Nature* **308**, 145-149 (1984).
10. Yanagi, Y., Chan, A., Chin, B., Minden, M. & Mak, T. W. *Proc. natn. Acad. Sci. U.S.A.* **82**, 3430-3434 (1985).
11. Kranz, D. M. *et al. Nature* **313**, 752-755 (1985).
12. Hayday, A. C. *et al. Cell* **40**, 259-269 (1985).
13. Heilig, J. S. *et al. Nature* **317**, 68-70 (1985).
14. Lefranc, M.-P. & Rabbitts, T. H. *Nature* **316**, 464-466 (1985).
15. Murre, C. *et al. Nature* **316**, 549-552 (1985).
16. Baer, R., Chen, K. C., Smith, S. D. & Rabbitts, T. H. *Cell* **43**, 705-713 (1985).
17. Smith, S. D. *et al. Cancer Res.* **44**, 5657-5660 (1984).
18. Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H. & Roe, B. A. *J. molec. Biol.* **143**, 161-178 (1980).
19. Staden, R. *Nucleic Acids Res.* **8**, 3673-3694 (1980).
20. Staden, R. *Nucleic Acids Res.* **12**, 521-538 (1984).
21. Karn, J., Matthes, H. W. D., Gait, M. J. & Brenner, S. *Gene* **32**, 217-224 (1984).
22. Vieira, J. & Messing, J. *Gene* **19**, 259-268 (1982).

An *Agrobacterium* transformation in the evolution of the genus *Nicotiana*

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Agrobacterium rhizogenes provokes neoplastic outgrowths on higher plants by transferring two small regions from the root-inducing (Ri) plasmid to the host genome¹⁻³. Homology with the left transferred DNA (T_L DNA) was detected in uninfected *Nicotiana glauca*¹ and a molecular clone (ANg31) of this region was obtained from *N. glauca*⁴. We report here that the genome of *N. glauca* contains a large inverted repeat which is homologous with the single copy T_L DNA of *A. rhizogenes*. Related sequences are present in several *Nicotiana* species, but are absent in others. The DNA sequence of the centre of the inverted repeat has been compared with the corresponding region from the bacterium. Both contain open reading frames which could potentially produce similar polypeptides. The data strongly suggest that an *Agrobacterium* infection resulted in genetic transformation early in the evolution of the genus *Nicotiana*.

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Fig. 1 a, Organization of the left T-DNA of plasmid pRiA4b. Indicated are: The approximate extent of plasmid DNA found in tumours³ (a break is indicated at the left end of the T-DNA as the left border varies but is usually left of this point). The positions and approximate sizes of transcripts are found in transformed *N. glauca* tissue⁵. The positions of the *rol* loci as defined by transposon mutagenesis³. These data are superimposed on an *Eco*RI and *Hind*III restriction map of this region. The extent of homology to the *N. glauca* genome and to λ Ng31 is indicated (region enclosed by broken lines). The *Eco*RI and *Hind*III map of the left arm of the *N. glauca* inverted repeat is shown at the bottom of the figure. In this presentation the left arm is shown in opposite orientation to *b*. *b*, A restriction map of the Ri-T_L homologous region of λ Ng31 compared with the left T-DNA of pRiA4b. The pRiA4b region is drawn as an imperfect inverted repeat with a breakpoint indicated by the dotted line. In the bacterium it is organized as *a*. Common restriction sites are shown above each map as arrows. Also indicated as hatched areas are the location of subcloned probes from these regions, g1 from λ Ng31 and r1 from pFW94². pFW94 is a cosmid clone including the left DNA of pRiA4b^{2,3}.

Methods. Restriction maps were made by single or multiple restriction enzyme digests of λ Ng31 or pFW94 or of subclones of these two. The alignment between the two maps was deduced from Southern blots of pFW94 probed with subclones of λ Ng31 and vice versa. Restriction digests were as manufacturer's instructions and Southern blots were as previously described¹². The clone g1 was subcloned from λ Ng31 DNA digested with *Xho*I into the *Sal*I site of pUC8¹³. The clone r1 was subcloned from pFW94 as a *Pst*I fragment in pUC8.

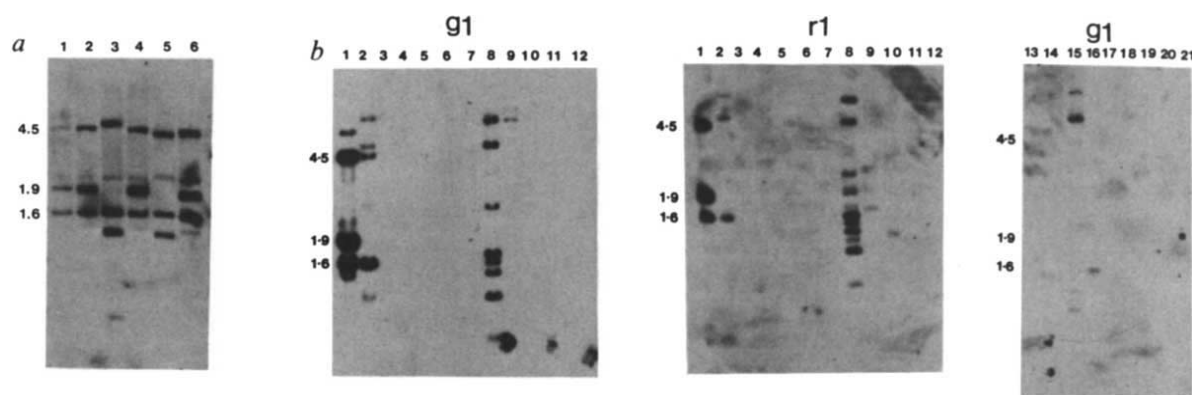


Fig. 2 a, Genomic Southern blots of *Eco*RI digest total DNA of six varieties of *N. glauca* hybridized with probe g1 from the Ri-T_L homologous DNA of *N. glauca*. The position and extent of g1 is indicated on Fig. 1b. Samples: 1, our standard line from which λ Ng31 was obtained; 2, line 23; 3, line 23a; 4, line 23b; 5, line 23c; and 6, line 23d. *b*, Genomic Southern blots of a variety of species from the genus *Nicotiana* and the family *Solanaceae* hybridized with either the left arm of the *N. glauca* inverted repeat, g1, or the corresponding region from pRiA4b probe r1 (positions indicated in Fig. 1b). Species are: 1, *N. glauca*; 2, *N. tabacum*; 3, *N. benthamiana*; 4, *N. debneyi*; 5, *N. langsdorffii*; 6, *N. glutinosa*; 7, *Solanum americanum*; 8, *N. otophora*; 9, *Petunia hybridia*; 10, *N. rustica*; 11, *Datura stramonium*; 12, *Solanum melongena* (Eggplant); 13, *N. knightiana*; 14, *N. benavidesii*; 15, *N. tomentosa*; 16, *N. tomentosiformis*; 17, *N. solanifolia*; 18, *N. cordifolia*; 19, *N. setchellii*; 20, *N. paniculata*; 21, *N. raimondii*. Control blots probed with the vector, pUC8 with no insert, showed no hybridization (data not shown). The 23 numbered *N. glauca* lines and some of the other tobaccos were obtained from V. A. Sissons, USDA, Agricultural Research Service, Tobacco Research Laboratory, Route 2, Box 16G, Oxford, North Carolina 27565, USA.

Methods. Southern blots were prepared as previously described¹³. The blots presented in panels *b* were prepared with the following modifications. Hybridization was performed in 50% formamide with $5 \times$ SSC at 42 °C. The blots were washed three times, for 30 min each, at 67 °C in $1 \times$ SSC.

The blots presented in panel *a* were prepared as described previously¹³. The final high stringency wash was omitted.



1562 AAAAGGTGTAAATTAATTTTCAGCAATAATTTACATCTTAATCATGAA ATAAAAAGCATCGTGACTGCGGCATCAAAACGCTTTGCCAATGACGCT TTT CAGGAACTCAATAGCGGGCACTTTAAGCTCTTACCCACCCCTCCCC 1714

 TTC GCG AAATCCAATAGCGGGCACTTTAAGCT TTAACCCACTCTCCCA 1575
 Hind III

1715 ATTATGGGATC...TTTGAAGATCCTTTCCTAAGGCAAAACAAAAAG ACAGAAAAAGAGCCCACTACGAAGAACCGTCAAGCATCAAGATGCA GTATTTTCACTTGATCCAGATTTAGGACT CCAATGCTTGAGCATCA 1861

 1576 ATTATGGGATCCTTTTTCATCTCTTTCGTAAGGCAAAACACACA CAAGAAAAAGCAAAACCACTACTGAACCCATGAACGATCAGCATGCA GTATTTTGGCTTGATCCAGATTTAAGGCTCCCGTCTTGCGCATGCA 1725

1862 TTTTATGGGATGTAATTAATTTTCAGATTTGCCCTGGCTTTCCGACCA GAGATTGTCTGATTTGGCGCACTTTCCGGTCCCATATTTAAATGGC ACTGGAACCTGCCATTTTTCACACTGTCCAACTCAGATTACAATGGCT 2011

 1726 TCTTGAT.....GTGATTGTGCAAACTACCTCTGGCTTTCCGACCA GAGACTGTCTGATTTGGCGCACTTTCCGACG CCGTATTTAAATGGC TGTGGCACTTGGC...TTTTCGTAACATCAACTCAGATCACAATGGAT 1866

rol B start
 BamHI

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 1867 CCCAAATGCTATCTCTCCAGATTTCAACGATAGATCTCACTCCAGC ATGGAGCCAGATAAATCTATTGAGGGGATCCGATTTGCTTTTGCATCT ATAGCCGTGACTATAGCAAAACCTCTGCTATTTCCAGAACGATGGCT 2016
 BamHI

Xho I

2162 GATGTACTCTTTGATTGGAGGAAAAATCTCTAGGATGATCTCTTAA ACATGGCTGAGCTTCTAAGAACAATACTGCTATCTCTCTATGT TTGTTGAGCAACAGATTGTGCTCGAAGGACCAACGCTGATTTATC 2311

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 Xho I

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 Pst I

Pst I

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 2617 AGTAAACCTGAAGAAAGAGGCTAAGCGCACTTGAATCCCTCGAGG CAACCTTTATCTATGATTTGCTAATAGTTCTATG...TACCCTCCGCGAG CTGTGACACAGAACCTTGGAGTTGAGCTACGTTGATGTTGATGAC 2765

rol B stop
 Pst I

2905 CTGTTTCTGTAAATAATTTTC ACACGTTAATCTCGCGCTGATTT TCGAAGACAGTGATA...TCATATAAATACTATAGACGATTCATCC ATATAAAAAAAGCTGCTAATACACTTGACTTTAAGATTGTCAACGTT 3049

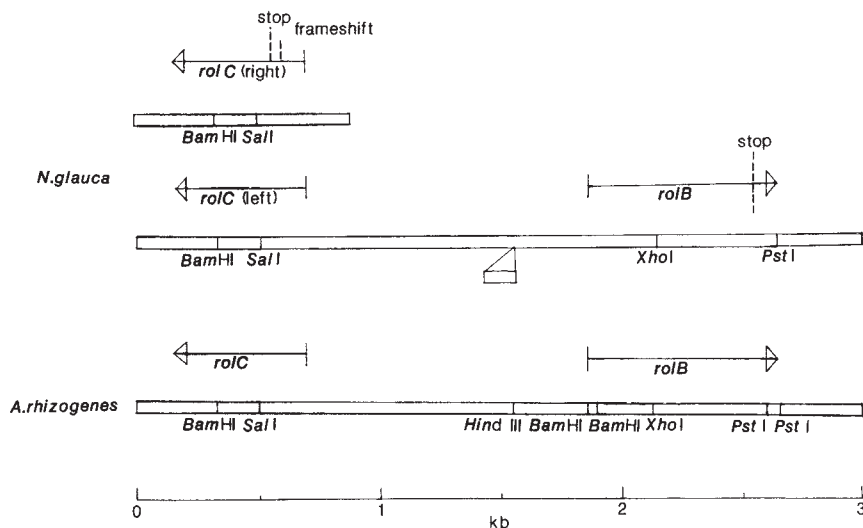
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Direct repeat Direct repeat

3051 ACATCTTAACAAACGATTTTTCAGACATTACAAAC 3086

 2914 TCAACAGTACATTTGACATAAAACATTTTCAGACAT 2950

Fig. 4 Comparison of the *rolB-C* region of *N. glauca* (top) and pRiA4b (bottom), based on the sequence data presented in Fig. 5. The left arm of the *N. glauca* structure is shown, 875 bp of the right arm is shown inverted and above the corresponding region of the left. The reading frames of pRiA4b and the corresponding regions from *N. glauca* are drawn as arrows. Interruptions in the *N. glauca* reading frames are indicated (dashed lines).



rol C

MET Ala Gly Val Asp Leu Cys Ala Leu Phe Phe Asn Leu Arg Val Lys Asp
 MET Ala Gly Asp Asp Leu Cys Ser Leu Phe Phe Lys Leu Lys Val Gly Asp
 Val Thr Ser Ser Asp Gly Leu Lys Lys His Ile Leu Ser Ala Ser Asp Gly Arg
 Val Thr Ser Ser Asp Gly Leu Ala Arg His MET Lys Asn Ala Ser Asn Gly Arg
 Asn Pro Leu Thr Gly Pro Gly Gly Asn Gln Ser MET Asp Val Asp Gly Gly Gly
 Lys Pro Leu Ile Gly Pro Gly Gly Asn Gln Ser MET Asp Ile Asp Gly Gly Gly
 Gly Thr Arg Asp Pro Gly Ile Leu Tyr Leu Tyr Val Asp Cys Pro Thr MET MET
 Gly Ser Val Gly His Gly Leu Leu Tyr Leu Tyr Val Asp Cys Pro Thr MET MET
 Gln Cys Phe Tyr Gly Thr Ser Phe Pro Tyr Asn Ser Arg His Gly Ala Leu Leu
 Leu Cys Phe Tyr Gly Gly Ser Leu Pro Tyr Asn Trp MET Gln Gly Ala Leu Leu
 Thr Asn Leu Pro Pro Tyr Gln Lys Asp Val Ser Leu Ser Gly Val Ser Arg Gly
 Thr Asn Leu Pro Pro Tyr Gln His Asp Val Thr Leu Asp Gly Val Asn Arg Gly
 Leu Arg Gln Ala Ser Gly Phe Phe Gly Tyr Gly Asp Pro Ile Arg Ser Ala Tyr
 Leu Arg Gln Ala Ser Gly Phe Phe Gly Tyr Ala Asp Pro MET Arg Ser Ala Tyr
 Phe Ala Ala Leu Ser Phe Pro Gly His Val Ala Lys Leu Asp Gly Gln MET Gly
 Phe Ala Ala Phe Ser Phe Pro Gly Arg Val Ile Lys Leu Asn Gly Gln MET Gly
 Leu Thr Ser Thr Asn Gly Gly Ser Leu Thr Phe Asp Leu Tyr Ala Ser Asp Gln
 Leu Thr Ser Thr Lys Gly Lys Cys Leu Thr Phe Asp Leu Tyr Ala Ser Thr Gln
 Leu Arg Leu Gly Pro Gly Ala Trp Val Arg His Gly Gly Cys Lys Phe Gly MET
 Leu Arg Phe Gly Pro Gly Gly Leu Val Arg His Gly Gly Cys Lys Phe Ala Ile
 Asn .
 Gly .

rol B

MET Ala Ser Gln Ser Gln Phe His Pro Arg Phe Gln Pro Arg Asn Leu Thr
 MET Asp Pro Lys Leu Leu Phe Leu Pro Arg Phe Gln Pro Val Asp Leu Thr
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 Tyr Ser Arg Asp Tyr Ser Lys Pro Leu Leu His Phe Gln Lys Arg Trp Ala Leu
 Val Ile Phe Asp Leu Gly Gly Lys Ser Leu Arg MET Asp Ile Leu Lys Gln Leu
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 Ala Gly Leu Leu Lys Asn Lys Ile Cys Tyr His Pro Pro MET Phe Val Gly Gln
 Ala Gly Leu Leu Lys Asn Lys Val Cys Tyr His Pro Pro MET Phe Val Ser Gln
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 Pro Asp Leu Ala Arg Gly Asn Asn Gln His Val Phe Val Tyr Leu Ser Arg Gly
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 Arg Val His Asn Leu Ala Trp Pro His Ser Arg Thr Gly Gly Pro Asp Leu Gly
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 Cys Phe Ile Ala Ile Phe Ala Ser Leu Phe Ile His Leu Leu Gly Leu Lys
 Val Thr Asn Leu Tyr Gly Arg Gly Val Asp Cys Thr Phe Phe Val Arg Arg Ala
 Val Thr Asn Val Tyr Gly Arg Gly Val Ala Cys Thr Phe Phe Leu Arg Arg
 Ser Thr Asn Arg Pro Tyr Asp Val Val Ala Phe Gly Thr Thr
 Thr Gly Asn Arg Pro Tyr Asp Val Val Ala Cys Gly Thr Thr Gln Phe Thr Lys
 Asn Ala Leu Gly Ile Ser Arg Pro Ala Ala Ser Ser Pro Gly Pro Asp Leu Thr
 Leu Arg Leu Ser Gly Pro Asp Gln Gly Gly Gly Gly Gly Val MET Lys Pro Ala
 Ala Val Asn Leu Lys Lys Gly Ala

Fig. 5 Predicted amino-acid sequence of the *rol* gene products: left panel, Ng *rolC* left (upper) and *RirolC* (lower), right panel, Ng *rolB* (upper) and *RirolB* (lower). Identical amino acids are underlined.

To learn more of the origin of the region, we included other members of the genus *Nicotiana* and the family Solanaceae in the study (Fig. 2b). Homology with both the left arm of the *N. glauca* inverted repeat, g1, and the corresponding *A. rhizogenes* subclone, rl, is limited to species in the subgenera *rustica* and *tabacum*. However, not all species in these two subgenera showed homology. *Nicotiana otophora*, *N. tomentosiformis*, *N. tomentosa* and *N. benavidesii* all show homology. Probes from both *A. rhizogenes* and *N. glauca* hybridized to similar bands in each species. No homology was detected in three members of the subgenus *petunioides*. Other members of the Solanaceae (Fig. 2b) and other higher plant genera (data not shown) did not hybridize to these probes. In at least one species, *N. tabacum*, a large region corresponding to the *rolB* and *rolC* loci, is absent (data not shown). The dispensability and limited distribution provide strong evidence for the hypothesis that the T_L -homologous region results from an *Agrobacterium* infection early in the evolution of the genus *Nicotiana*.

We propose that early in the evolution of the genus *Nicotiana* an infection by *Agrobacterium* resulted in the introduction of a T-DNA closely resembling T_L DNA of pRiA4b into the plant genome. This event may not have resulted in neoplastic growth because present-day Ri- T_L DNA is insufficient for rhizogenesis on any host tested³. However, even if neoplastic growth resulted, *A. rhizogenes*-transformed tissue can revert to normal growth both *in vitro* and *in vivo*^{7,8}. We further propose that flowering shoots derived from the transformed tissue were fertile and gave rise to a transformed progeny. This mechanism is plausible as *A. rhizogenes*-transformed plants are fertile and transmit T_L DNA to their progeny^{7,8}. Subsequent evolution of the transformed plants gave rise to the subgenera *rustica* and *tabacum*. The data suggest that this infection occurred in the species complex preceding the divergence of pre-*tabacum* and pre-

rustica lines. Species lacking detectable homology may result from subsequent loss of these sequences. An alternative possibility is a later origin and lateral spread through these two subgenera by interspecific hybridization.

The sequence of the plant and plasmid copies of *rolB-C* region are presented and compared in Fig. 4. Only two open reading frames >300 base pairs (bp) were found in the 3-kb region of pRiA4b sequence. The positions correspond to the locations of *rolB* and *rolC* as identified by transposon mutagenesis and to the approximate position and sizes of messages (compare Figs 3 and 4 and Fig. 1a). We designate these *A. rhizogenes* reading frames as *RirolB* and *RirolC*, to distinguish them from the corresponding regions from *N. glauca*, *NgrolB* and *NgrolC*.

The corresponding region of the *N. glauca* T_L homologous region is the centre of the imperfect inverted repeat. In this presentation the sequence of the long left arm is shown up to the break point of the inversion (Fig. 4). We sequenced 875 bp of this right arm and compared it with the left (Fig. 4). Since the generation of the inverted repeat, the two arms have diverged by 1.5%. Within the sequenced regions each arm of the inverted repeat is approximately colinear with the corresponding *A. rhizogenes* sequence. We examined the *N. glauca* sequence for reading frames corresponding to those found in *A. rhizogenes*. Because of the imperfect inverted repeat, two regions corresponding to *rolC* are present in *N. glauca*. The *rolC* region of the right arm of the inverted repeat, *NgrolC* right, is a pseudo-gene. The reading frame corresponding to *RirolC* is interrupted by a 1-bp pair deletion only 100 bp into the gene and 32 bp later a substitution generates a stop codon in the original reading frame. The *NgrolC* left reading frame is intact and translation start and stop codons are present at the corresponding positions to *RirolC*. The *NgrolB* reading frame starts at the corresponding base and continues for 633 bp, 144 bp shorter than *RirolB*. *NgrolB* has an early stop codon compared

with *RiolB* (Figs 4, 5). Thus, *NgrolB* can potentially encode a polypeptide 48 amino acids shorter than *RiolB* (Fig. 5). The sequence of *NgrolB* beyond the early stop is strongly homologous with *RiolB*. The above considerations reveal that *N. glauca rolB* and *rolC* genes are at least partially degenerate in that *rolB* is truncated and *rolC* right has both a frameshift and a stop codon. As *NgrolC* left is intact and *NgrolC* right is not, it is reasonable to assume their common ancestral sequence resembled *NgrolC* left at these positions. All five reading frames are preceded by a sequence similar to a TATAA box and all show a consensus eukaryotic ribosome binding site of the form A/GXXATGG⁹.

Since the divergence of these *A. rhizogenes* and *N. glauca* sequences, only a single frameshift has occurred in 1,857 bp of reading frame. In contrast, 25 frameshifts are evident in 1,240 bp of homologous intergenic DNA. This difference implies that selection has maintained the open reading frames in both sequences. The reading frames are 83% homologous in DNA sequence, whereas the homology in the intergenic regions averages only 75%, not counting frameshifts and not including obviously non-homologous regions. Comparing *NgrolC* left and *RiolC*, the number of changes at the third base of the codon exceeds the sum of such changes at the first and second. The amino-acid sequences of the predicted polypeptides are 75% homologous (Fig. 5). Both sets of reading frames have been conserved in length and content for at least some of the period of time since their divergence.

The Ri-T_L homologous region found in *N. glauca* is a large, imperfect, inverted repeat. Similar structures can be generated on integration of T-DNAs⁶. None of the *A. rhizogenes* strains examined to date are duplicated in this region^{2,3,10,11}. Therefore, the inverted repeat was probably generated in the genus *Nicotiana* either at integration or by a later duplication. The levels of sequence divergence between the left and the right arms can be used to set a minimum estimate on the divergence since the original integration event. The sequence divergence between the left and right arms is 1.5% and for the *rolC* region alone is 1.9%, whereas the divergence between *RiolC* and either *NgrolC* is 17%. Most of the divergence preceded duplication of the *N. glauca* region and may have occurred in the *Agrobacterium* strain inciting the infection. If duplication occurred at infection, >85% of the divergence would have been in separate *Agrobacterium* strains. It seems possible that *A. rhizogenes* strains more related to the inciting bacterium than pRiA4b exist.

The left arm of the *N. glauca* inverted repeat contains an intact *rolC* reading frame and a *rolB* reading frame nearly 80% of the length of the *A. rhizogenes rolB* reading frame. The integrity of these reading frames might be taken as evidence for conservation, and thus function, in the genus *Nicotiana*. However, most of the divergence preceded duplication of this region and may have occurred in *Agrobacterium* where selective pressure would maintain these reading frames. If this is true, the presence of these reading frames in *N. glauca* may merely reflect the passage of insufficient time to allow them to become obviously degenerate.

We have presented evidence for a lateral interkingdom transfer of DNA from *Agrobacterium* to *Nicotiana* and its subsequent passage in evolutionary time. Plants transformed with the T_L DNA of present-day strains of *A. rhizogenes* show various developmental changes, including reduced apical dominance, wrinkled leaves and an increased width-to-length ratio in leaves⁸. These phenotypes are presumably caused by the expression of the integrated *rol* genes. The presence of comparable reading frames in the genome of *N. glauca* may have in evolution and may now significantly contribute to phenotype of plants containing them.

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- White, F. F., Ghidossi, G., Gordon, M. P. & Nester, E. W. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3193-3197 (1982).
- Huffman, G. A., White, F. F., Gordon, M. P. & Nester, E. W. *J. Bact.* **157**, 269-276 (1984).
- White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P. & Nester, E. W. *J. Bact.* **164**, 33-44 (1985).
- White, F. F., Garfinkel, D. J., Huffman, G. A., Gordon, M. P. & Nester, E. W. *Nature* **310**, 348-350 (1983).
- Taylor, B. H., White, F. F., Nester, E. W. & Gordon, M. P. *Molec. gen. Genet.* (in the press).
- Kwok, W. W., Nester, E. W. & Gordon, M. P. *Nucleic Acids Res.* **13**, 459-471 (1985).
- Costantino, P., Spano, Pomponi, M., Benvenuto, E. & Ancora, G. *J. Molec. appl. Genet.* **2**, 465-470 (1984).
- Tepfer, D. *Cell* **37**, 959-967 (1984).
- Barker, R. F., Idler, K. B., Thompson, D. V. & Kemp, J. D. *Pl. molec. Biol.* **2**, 335-350 (1983).
- Lahners, K., Byrne, M. C. & Chilton, M.-D. *Plasmid* **11**, 130-140 (1984).
- Spano, L., Pomponi, M., Costantino, P., Van Slogteren, G. M. S. & Tempe, J. *Pl. molec. Biol.* **1**, 291-300 (1982).
- Thomasow, M. F., Nutter, R., Montoya, A. L., Gordon, M. P. & Nester, E. W. *Cell* **19**, 729-739 (1980).
- Vieira, J. & Messing, J. *Gene* **19**, 259-268 (1982).
- Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).
- Guo, L.-H. & Wu, R. *Nucleic Acids Res.* **10**, 2065-2084 (1982).

Erratum

Mycobacterium leprae-specific protein antigens defined by cloned human helper T cells

T. H. M. Ottenhoff, P. R. Klatser, J. Ivani, D. G. Elferink, M. Y. L. de Wit & R. R. P. de Vries

Nature **319**, 66-68 (1986).

IN this letter there were errors in the Note Added in Proof, which which should read: Recent experiments show that also on the recombinant 36K *M. leprae*-specific protein, expressed in *Escherichia coli*, different *M. leprae*-specific protein determinants are detected by these *M. leprae*-specific TLC.

Corrigendum

Efficiency of petroleum expulsion from shale source rocks

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Nature **311**, 745-748 (1984).

AS discovered only recently, the squalane product which we used as internal standard for quantitation of the gas chromatography data was contaminated by 5.4% pristane. Therefore, the data based on pristane concentrations shown in Figs 2 and 3, respectively, are in error. Values were corrected accordingly and selected rock samples re-analysed for verification. Listed are sample depth (m); the corrected pristane/*n*-heptadecane ratio; and, in parentheses, the pristane/phytane ratio: 1,126.7 = 0.60 (3.21); 1,131.3 = 0.44 (2.95); 1,132.6 = 0.58 (2.65); 1,133.1 = 0.48 (2.50); 1,135.3 = 0.29 (1.60); 1,142.8 = 0.21 (1.96); 1,148.1 = 0.14 (1.82); 1,152.65 = 0.19 (1.96); 1,155.5 = 0.11 (1.58); 1,159.5 = 0.06 (2.14); 1,161.0 = 0.06 (1.26); 1,162.5 = 0.05 (1.16).

As seen from these corrected data, our earlier conclusion of an increasing fractionation between pristane and *n*-heptadecane (expressed by the increase of the ratio between both compounds) with increasing degree of hydrocarbon expulsion from shale source rocks remains valid. However, the magnitude of this fractionation and the corresponding ratio changes are considerably less than we previously thought.

Palaeontological and molecular views of panda phylogeny

THE recent valuable contribution to panda phylogeny by O'Brien *et al.*¹ corroborates quite well the existing palaeontological information.

Ailuropoda, the giant panda, has distinctive teeth and skull. It was a major and widespread element in the early Pleistocene fauna of southeast Asia, and its ancestors occurred much farther afield. Thenius^{2,3} has shown that it originated from the poorly known late Miocene bear *Agriarctos*, which has not yet been found outside Europe. *Agriarctos* had a separate origin from the modern bears in the earlier Miocene genus *Ursavus*. Thenius put the divergence time of *Ailuropoda* between 15 and 20 Myr, as does an average of the molecular chronologies of O'Brien *et al.*, although neither approach can as yet be very accurate here.

Thenius² and others^{4,5} have also contributed to the fossil history of *Ailurus*, the lesser panda. The Holarctic Pliocene genus *Parailurus* is a collateral relative, and both came from the Eurasian genus *Sivanasua* of the early and middle Miocene. *Sivanasua* is a good if primitive procyonid, branching between the divergence of modern mustelids and procyonids. The Procyonidae would perhaps be polyphyletic if the ancestral musteloids are excluded from this family; even *Ailurus* retains some basicranial and other features changed in other modern procyonids but also retained by bears. The relevant late Eocene and Oligocene genera of the Amphicyonidae, in a broad sense, have not yet been studied adequately by current methods and so the time of divergence of Procyonidae and Ursidae within that interval is unclear. I agree^{1,6} that subfamilial distinction of each panda, with its relatives, from the rest of the members of their respective families is appropriate.

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- O'Brien, S. J., Nash, W. G., Wildt, D. E., Bush, M. E. & Benveniste, R. E. *Nature* **317**, 140-144 (1985).
- Thenius, E. Z. *Säugetierk.* **44**, 286-305 (1979).
- Thenius, E. Z. *Geol. Wiss.* **10**, 1029-1042 (1982).
- Pilgrim, G. E. *Palaeont. Indica, New Ser.* **18**, 1-232 (1932).
- Tedford, R. H. & Gustafson, E. P. *Nature* **265**, 621-623 (1977).
- Stains, H. J. in *Orders and Families of Recent Mammals of the World* (eds Anderson, S. & Jones, J. K., Jr) 491-521 (Wiley, New York, 1984).

O'BRIEN *ET AL.* REPLY—We are pleased indeed to have our molecular phylogeny corroborated by the palaeontological record as pointed out by such eminent authorities as Professors Kurtén (*Nature* **318**, 487; 1985) and Van Valen (above). It is reassuring to find that the molecular

topology we have constructed can be consistently interpreted by the limited, but informative fossil record of the ursid and procyonid progenitors. Because of inherent limitations in any individual approach, synthetic analysis of multiple methods of phylogenetic inference is particularly important in the resolution of evolutionary relationship. Derivation of a consensus molecular / cytogenetic / palaeontological story for the pandas is a persuasive example of how a long debated puzzle can be correctly resolved and the bases for the controversies themselves ultimately interpreted. I hope that our agreement concerning the assignment of subfamily status of the giant panda in the Ursidae will be adopted by students of mammalian taxonomy.

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Oncofetal antigens

IN the review article by Feizi¹ the term 'oncofetal' antigen (OFA) is used to describe those determinants that are expressed in fetal, tumour and specific adult tissues in modified or quantitatively varying levels. This misnomer has become a common problem to many tumour biologists. Many view the oncofetal antigen prototype as carcinoembryonic antigen (CEA) or α -fetoprotein (AFP). This is incorrect. Although these antigens were first considered as examples of re-expressed fetal gene products that were proposed to be tumour-specific in adults, the subsequent discovery of their presence in diseased, non-tumour tissue as well as some limited expression in normal adult tissues should have caused them to be classified as differentiation antigens. The purpose of this response is to implore tumour biologists to use the correct nomenclature.

For clarification, the original definition of the term oncofetal antigen, put into contemporary use in the early 1970s by Alexander, Medawar and my group after CEA and AFP were found not to be tumour-specific markers, was as follows: OFAs are antigen substances expressed exclusively as phase-specific autoantigens in developing embryo or fetal tissues of metazoans and in their tumours². The important criteria that must be fulfilled if an epitope is to be classed as an OFA are:

- (1) The expression of the OFA epitope must be restricted to embryo, fetal and tumour tissue and not be found in any adult, normal tissue;
- (2) The OFA epitope must be autoantigenic in the pregnant female carrying the embryo or fetus and in the tumour-bearing host.

Epitopes not fulfilling these criteria are differentiation antigens if they are transiently or permanently expressed in adult as well as fetal and/or tumour tissue with or without autoimmunogenicity and reflect various stages of cellular differentiation.

Adherence to this simple set of distinguishing criteria in naming tissue-associated antigens will greatly avoid confusion. Examples of possible true OFAs are the polypeptides of relative molecular mass 44,000 and 200,000 recently described in fetal and tumour cells of rodents and man³ and possibly certain phosphorylated or truncated forms of the protein p53.

I further recommend that antigens similar to those described in ref. 1 be designated embryo/fetal/tumour (EFT) differentiation antigens. Such EFT differentiation determinants would conventionally be detected with xenogeneically derived antibody or monoclonal antibody as epitopes that appear in greater concentration in embryo-fetal and tumour tissue than in normal, fully differentiated, adult tissue. The term oncodevelopmental antigen should be discontinued as it has never been formally defined.

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- Feizi, T. *Nature* **314**, 53-57 (1985).
- Ambrose, K., Andersson, N. & Coggin, J. *Nature* **233**, 194-195 (1971).
- Payne, W. & Coggin, J. H. *J. natn. Cancer Inst.* **75**, 527-544 (1985).

FEIZI REPLIES—My article¹ was based on information available on the precise antigenic determinants expressed on the surface of cells and recognized by monoclonal antibodies as tumour-associated, developmentally regulated or other differentiation antigens. At the time (and to my knowledge even now) the only characterized determinants have been carbohydrate sequences. Clearly it will be desirable to classify properly the various antigens in their biological context when their structures and distributions in diverse tissues have been thoroughly evaluated. Nomenclature can then be rationalized.

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Automated continuous flow peptide synthesis

From R. P. Andrews

As peptide-based drugs and reagents become a reality, a new method of peptide synthesis is beginning to come into its own.

THE chemical synthesis of peptides is a rapidly expanding feature of biotechnology, urged on by the two areas of research that stand to benefit from it. In immunology, detailed antigen-antibody studies consume large numbers of peptides, and analogues of a single parent sequence are often needed in smaller quantities. In pharmaceutical research, short peptide fragments mimicking fragments of viral protein are the key components of synthetic vaccines¹.

Synthetic peptides can be genetically engineered, but this process involves a large initial capital outlay and is generally unsuitable for producing small amounts of short (less than 30 amino acids) peptides for research use. Attention has therefore focused on ways of improving the chemical methods for peptide synthesis. Within the past few years, a novel and superior chemistry has emerged.

Tradition

Merrifield first introduced the solid-phase method of peptide synthesis in 1962². Initially, an ester linkage attaches the C-terminal amino acid to a polystyrene support. Subsequent amino acids are added by a repetitive protocol of: (1) amino protecting group cleavage by trifluoroacetic acid (TFA); (2) neutralization using triethylamine/dichloromethane (DCM); and (3) acylation/amino-acid addition, usually using *t*-butoxycarbonyl (Boc) amino acids in the presence of dicyclohexylcarbodiimide (DCCI) and DCM.

Anhydrous hydrogen fluoride breaks the ester linkage to the polystyrene support and removes the side-chain protecting groups from those amino acids with reactive side chains.

Although the Merrifield chemistry has been used successfully for 20 years, it does have drawbacks. The use of TFA at every addition cycle and the use of hydrogen fluoride for removing the peptide from the resin can cause side reactions with many amino acids that are unstable in vigorous acidic treatment³⁻⁶. The products of acid-catalysed cleavage reactions can cause additional problems; for instance, benzyl cations can react with the nucleophilic sulphur atom of methionine or modify tyrosine's phenolic ring. The repeated use of strong acids can also cause selective peptide bond cleavage — aspartyl-prolyl bonds are particularly susceptible⁷.

Overall, the Merrifield method often achieves high levels of protein purity after a final purification by HPLC. Purification

of the final synthesis product, however, still poses difficulties, as even the most sophisticated HPLC systems may not separate peptide impurities that are of marginally different length and composition.

Polyamide method

At the Medical Research Council (MRC) in Cambridge, England, workers have devised a new method of peptide synthesis that eliminates many of the customary problems and could eventually allow full control of the synthesis reaction⁸⁻¹⁰. Based on polyamide resin rather than polystyrene, this solid-phase system conducts all the chemical operations under mild and substantially optimized reaction conditions. The method still adheres to the basic principles laid down by Merrifield, but differs from the established technique in several ways.

First, the substituting of polystyrene with polar polyamide gel supports enables polar aprotic solvents such as dimethylformamide (DMF) to freely permeate the gel media. Second, the use of fluorenylmethoxycarbonyl (Fmoc) amino-acid derivatives¹¹, rather than Boc derivatives, removes the requirement for repeated acid treatments at each deprotection reaction. Third, instead of a simple benzyl ester agent for peptide-resin linkage, the polyamide method uses a range of substituted

dilute acid or basic conditions¹².

Perhaps the main advantage of the polyamide method is its suitability for use in a continuous flow system. The introduction of a composite resin¹³ is the key to this method's success. In this resin, the dimethylacrylamide-based monomer mixture is polymerized within the pores of a rigid, macroporous, kieselguhr matrix. Reactant diffusion into and out of this matrix is rapid and pressure evolution is negligible under normal flow conditions. Of equal importance, flow systems are more amenable to analytical control. Continuous spectrophotometric effluent monitoring is easily achieved and raises the possibility of automated feedback control of each synthesis cycle.

The chemical character of the amino-acid derivatives figures importantly in schemes for true synthesis automation. For polyamide chemistry, Fmoc amino-acid symmetric anhydrides have been the derivatives of choice, but these unstable compounds must be formed immediately prior to synthesis. The introduction of Fmoc amino-acid pentafluorophenyl (PFP) active esters combines the high reaction rates and freedom from side reactions achievable with Fmoc amino-acid anhydrides with the crystallinity and stability of active esters¹³. Full automation of the Fmoc polyamide method is then relatively simple.

The synthesis protocol in conjunction with Fmoc amino-acid PFP esters is shown in Fig. 1. The first amino-acid derivative is secured with the formation of an ester bond using 4-dimethylaminopyridine (DMAP) in DMF. Deprotection of the N-terminal amino-acid group is rapidly achieved using 20 per cent piperidine in DMF. Subsequent amino-acid derivatives are then added in DMF with 1-hydroxybenzotriazole (HOBt) acting as a catalyst, and each N-terminal amino-acid group must be deprotected before the next amino-acid is added. The protocol shown in Fig. 1 incorporates an acid-labile peptide-resin linkage agent which is finally removed in 95 per cent TFA.

In 1983, Sheppard described a semi-automated instrument for peptide synthesis using the polyamide protocol⁹. In collaboration with the MRC in Cambridge, LKB Biochrom has now developed a fully automated system.

The commercial instrument closely follows the original design⁹. A single amino-acid addition (except for the C-terminal residue) follows the sequence:

(1) The autoinjector draws a mixture of

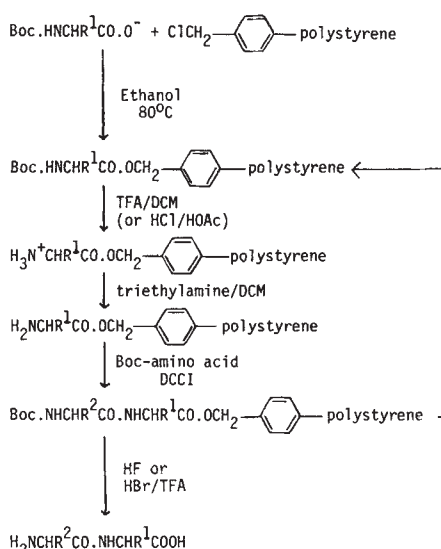


Fig. 1 Protocol for peptide synthesis using the polyamide method and Fmoc amino-acid PFP esters. Norleucine (Nle) is permanently bound to the polymer and acts as an internal reference.

benzyl alcohol derivatives that can be cleaved under either strong acid (TFA),

amino-acid derivative and HOBt catalyst dissolved in DMF and the mixture is delivered to the column via the spectrophotometer. The instrument then enters a recirculation mode. During the first pass of the amino-acid derivative through the column, 90 to 95 per cent of the amino groups available are acylated⁹. Subsequent passes usually complete acylation in 30 min.

(2) Unreacted Fmoc amino-acid PFP ester is pumped to waste and the column is washed with DMF.

(3) To deprotect the N-terminal amino-acid, 20 per cent piperidine in DMF is pumped through the column.

(4) All traces of piperidine are removed from the column with a DMF wash cycle, which prepares the column for the next amino-acid addition. A complete cycle takes approximately one hour.

Cycle control must be accurate and flexible, a personal computer is required

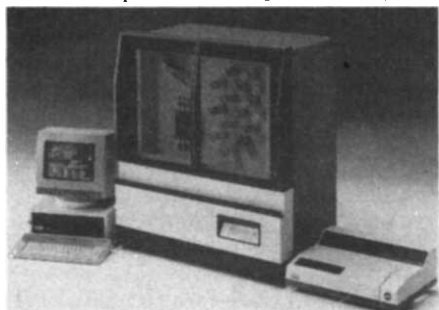


Fig. 2 LKB's fully automated continuous-flow peptide synthesizer

to store and execute synthesis protocols. The computer enables the user to change synthesis parameters such as acylation or deprotection times.

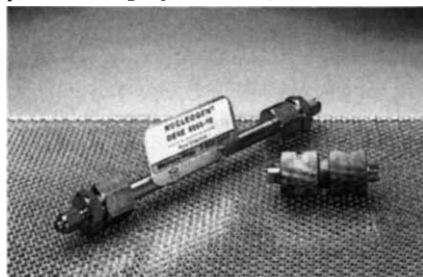
The complete peptide synthesis system is shown in Fig. 2. HPLC data from unpurified cleaved peptides have revealed excellent purity levels¹⁴, indicating that once continuous-flow polyamide synthesis has been fully developed the purification step may become unnecessary. □

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1. Audibert, F. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 5042 (1982).
2. Merrifield, R.B. *Fedn Proc.* **21**, 41 (1962); *J. Am. chem. Soc.* **85**, 2149 (1963).
3. Sano, S., & Kawanishi, S. *Am. chem. Soc.* **97**, 3480 (1975).
4. Feinberg, R.S. & Merrifield, R.B. *J. Am. chem. Soc.* **97**, 3485 (1975).
5. Brandt, W.F., Henschen, A. & Van Holt, C. in *Methods in Protein Sequence Analysis* (ed. Elzinga, M.) 101-109 (Humana Clifton, New Jersey, 1982).
6. Ondetti, M.A., Deer, A., Sheenan, J.T., Pluscic, J. & Kocy, O. *Biochemistry* **7**, 4069 (1968).
7. Wade, J.D., Fitzgerald, S.P., McDonald, M.R., McDougall, J.G. & Treger, G.W. *Biopolymers* (in press).
8. Atherton, E., Gait, M.J., Sheppard, R.C. & Williams, B.J. *Bioorgan. Chem.* **8**, 351-370 (1979).
9. Sheppard, R.C. *Chem. Brit.* **19**, 402-414 (1983).
10. Atherton, E., Clive, D.L.J. & Sheppard, R.C. *J.C.S. Perkin* **529**-537 (1981).
11. Atherton, E., Logan, C.J. & Sheppard, R.C. *J.C.S. Perkin* **1529**-537 (1981).
12. Sheppard, R.C. & Williams, B.J. *Int. J. Peptide Protein Res.* **20**, 451-454 (1982).
13. Atherton, E., Brown, E. & Sheppard, R.C. *JCS Chem. Commun.*, 1151-1152 (1981).
14. Auffret, A.D. & Meade, L.G. *Proc. Synth. Peptides Biol. Med.*, Hämeenlinna, Finland 6-8 June 1985 (in press).

A separation sampler

Innovations in separation methods dominate the new products for peptide and DNA research. Machery Nagel has introduced a new series of **anion-exchange HPLC columns** that will separate compounds ranging from oligonucleotides to



Nucleogen columns work from pH 2.5 to 8.0

very large DNA molecules. (Reader Service No. 100). The Nucleogen stationary phase series consists of three groups of columns based on silica gel with pore sizes of 60 Å, 500 Å and 4,000 Å. The Nucleogen DEAE 60-7 column is designed for the isolation of short nucleic acid chains (MW 25,000 to 1,000,000), while the DEAE 4000-10 performs separations of high-molecular weight nucleic acids. The columns operate at high flow rates and high pressures (up to 300 bar) and will survive 1,000 repetitions without loss of resolution. In the United Kingdom these new columns are available from Technicol Ltd.

The Separations Group is offering a high-efficiency **HPLC column for rapid nucleotide analysis** (Reader Service No. 101). Using a low-capacity ion-exchange

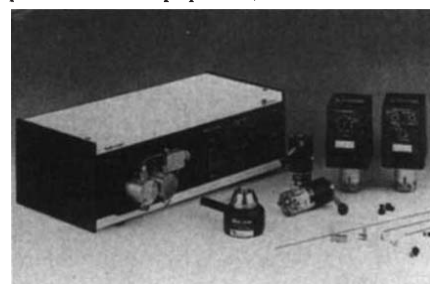


The Vydac improves upon traditional HPLC

material on a protected silica substrate, the Vydac nucleotide analysis column can resolve the twelve major mono-, di- and triphosphonucleotides completely in 10 minutes. It measures 5 cm in length with a 4.6 mm internal diameter, and supports a flow rate of 2.0 ml per minute.

For **preparative and process HPLC**, Separations Technology Inc. has produced three new instruments able to provide both isocratic and continuous gradient solvent delivery (Reader Service No. 102). Because all three systems feature hydraulic dual-head pumps for high-pressure operation, they can be packed with particles as small as 10 µm in diameter to achieve optimum resolution. The versatile ST/LAB 800 utilizes 1- to 4-inch diameter columns and performs at pressures up to 1,800 p.s.i. It accommodates a sample load of up to 250 g and has a maximum flow rate of 1,600 ml per minute. The ST/LAB 2000 provides pilot-plant level HPLC for sample loads ranging from 50 to 1,000 g per minute. The unit has a throughput of up to 3,000 ml per minute on 1- to 6-inch diameter columns. Completely explosion-proof, the ST/LAB 2000 and the process-scale ST/Process 2000 systems operate at pressures up to 2,000 p.s.i. The ST/Process 2000 will separate kilogram-size sample loads on 4- to 10-inch diameter columns with a maximum throughput of 8,500 ml per minute.

For high-pressure separation of active proteins and peptides, Pharmacia Inc.



Pharmacia's system preserves bioactivity

makes fully **biocompatible FPLC systems** supplemented by a new line of components (Reader Service No. 103). The new FPLC instruments extend the system's solvent delivery capacity to 5,100 p.s.i. and can construct configurations for both isocratic and gradient separations. The centre of the expanded solvent delivery system, the high performance pump P-3500, produces highly consistent flow rates with low pulsation. The low dead volume mixer, model 35 MPa, and the PV-7 valve or PMV-7 motor valve comprise the remainder of the new system. The 7-port valves enable manual or automatic sample injection, respectively. The tubing kit, P-3500, connects the instruments with tubings, fittings and unions made from inert materials.

The first **phenyl-bonded reversed-phase column** of polymer base gel comes from Kratos Analytical (Reader Service No. 104). Highly concentrated phenyl groups

These notes are based on information provided by the manufacturers. To obtain further details about these products use the reader service card bound inside the journal.

give this new column the capacity to separate proteins and biological polymers. The average pore size of the Toyo Soda TSKgel Phenyl-5PW RP is about 100 nm.

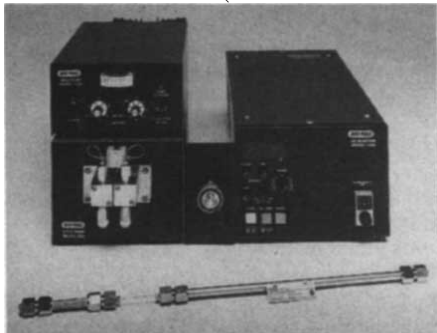


Kratos columns are packed with two new gels

It boasts outstanding chemical stability and separation of high-molecular-weight proteins over a wide pH and solvent range. Kratos also supplies a **silanol-free stationary phase column** that performs separations not previously possible with reversed-phase columns with a minimal amount of organic solvent. The C₁-bonded silica gel gives a high recovery rate and low adsorptivity, and the TSKgel TMS-250 is best suited to analytical and preparative chromatographic resolution.

Interaction Chemicals Inc. has developed a **novel material for reversed-phase liquid chromatography** applications. The ACT-1 column contains Macrophase-1, a polymer that has been covalently bonded with $-(CH_2)_{17}CH_3$, or 'C18', functional groups (Reader Service No. 105). Because only carbon-carbon and carbon-hydrogen bonds exist in the polymer matrix, the material remains stable from pH 0 to 14. The C18 groups reduce capacity factors dramatically, allowing separation of aliphatic and heterocyclic amines. The ACT-1's rigid structure (80 Å pore size) holds up under normal HPLC conditions, and its pH stability facilitates the separation of nitrogen-containing compounds.

For fast protein profiling, the Bio-Rad Quick Check analyser gives **HPLC results in 15 to 20 minutes** (Reader Service No.



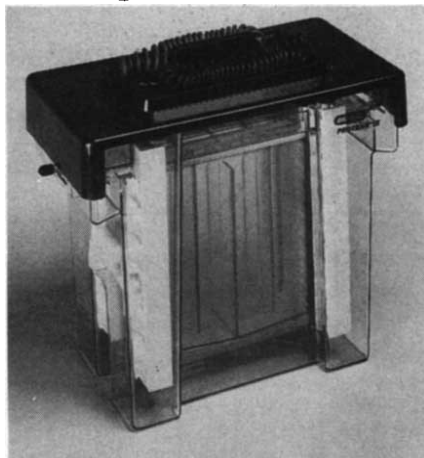
The Quick Check gives optimum results between pH 2.0 and 7.0

106). The instrument can monitor protein purification steps or enzymatic reactions, and check the purity of individual proteins. It separates proteins by size with an

efficiency and gentleness comparable to that of classical gel filtration chromatography. The analyser's buffers will not denature samples, so fragile molecules can be recovered intact for further analysis and characterization.

Du Pont has introduced a new addition to its **gel filtration** family for separating proteins by size. The Zorbax GF-450 Bio Series column performs separations of biomolecules with molecular weights from 17,500 to 900,000 with high resolution, outstanding reproducibility and durability over a broad pH range (Reader Service No. 107). As with other Bio Series columns, the typical protein recovery rate of the GF-450 is 95% or greater, and biological activity is generally maintained. The column will carry out lengthy separations in aqueous mobile phases having pH values up to 8.5. The GF-450 is 25 cm long and has an inner diameter of 9.4 mm.

Electrophoresis extras



Bio-Rad's Protean II is a snap to assemble

Several manufacturers offer other **improvements in electrophoresis equipment**. Bio Rad's versatile Protean II slab cell (Reader Service No. 108) can accommodate most common electrophoretic techniques, including SDS, two-dimensional, native and agarose gel electrophoresis. Supplemented with various combs, spacers and accessories, the Protean II can run up to four slab gels or 16 tube gels simultaneously. The basic unit handles gels 16 or 20 cm long, and requires only 1.5 litres of buffer. The central cooling core of the Protean II cell assures even heat distribution over the entire gel length, and single-screw sandwich clamps provide even pressure distribution.

For electrophoretic separation of DNA and RNA fragments in horizontal agarose gels, LKB-Produkter AB now produces **complete agarose submarine systems** based on the new Miniphor and Maxiphor units (Reader Service No. 109). The Maxiphor system is intended for preparative and analytical separations and is compati-



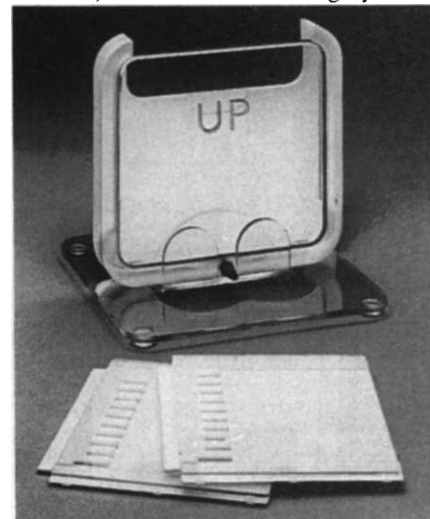
LKB designed two new agaroses for these submarine units

ble with any gel not greater than 10 mm thick, 15 cm wide and 25 cm long. The Miniphor system performs rapid analytical separations. Both units include the MacroDrive I power supply and are accompanied by a MacroVue ultraviolet transilluminator for visualizing fragments stained with ethidium bromide.

Four **calibration protein kits** are now available from Boehringer Mannheim Biochemicals (Reader Service No. 110). The kits supply individually packaged, highly purified and carefully selected proteins for even band distribution and equal band intensity. Two of the kits are applicable for molecular sieve gel chromatography, the other two for SDS gel electrophoresis.

Gradients, home-made or ready-made

As an accessory to the Mighty Small I and II vertical slab gel units, Hoefer Scientific Instruments now offers a **Mighty Small dual gel caster**, SE 265 (Reader Service No. 111). Included in the Mighty Small



Hoefer's gel caster sets two 7 × 8 cm gels

package are 10 glass plates, two alumina plates, an acrylic stand, 100 sheets of waxed paper and four of Hoefer's T-shaped spacers.

Integrated Separation Systems details their line of pre-cast, ready-to-use 1.5 mm

thick **gradient polyacrylamide gels** in their Sepragels brochure (Reader Service No. 112). The gels are ideal for silver staining and two-dimensional analysis. Sepragels come in heavy-metal-free glass cassettes with glass spacers, and fit all large-gel Hoefer, Bio-Rad, LKB and Pharmacia vertical devices using cassettes in which both glass plates are flush across the top.

A new arrival in **HPLC gradient systems**, Life Science Laboratories Ltd's low-pressure gradient module S8110 can produce binary and ternary gradients with a reproducibility at least as good as that achieved by high-pressure methods (Reader Service No. 113). Gradient profiles made up of two or three solvents can be mixed with 1% accuracy. Interfaced to the S2000 microprocessor-based controller, the S8110 creates a gradient by the selective switching of two-way solenoid valves attached to pressurized reagent bottles. The valves and mixing chambers are designed to eliminate temperature effects, and all components that come into contact with the solvents are composed of inert materials.

Quick and easy cartridges

Romicon, Inc. is ushering in a **new series of disposable cartridges** under the Romi-



The Romisorb resin features 780 square metres of surface area

sorb title (Reader Service No. 114). The Romisorb SP cartridge comes prepacked with Rohm and Haas's macroreticular copolymer resin, Amberlite XAD-4. This non-ionic resin is suitable for sorbate with MW below 1,000. It adsorbs via van der Waals' interactions, so lends itself to the adsorption of molecules with aromatic rings or aliphatic tails.

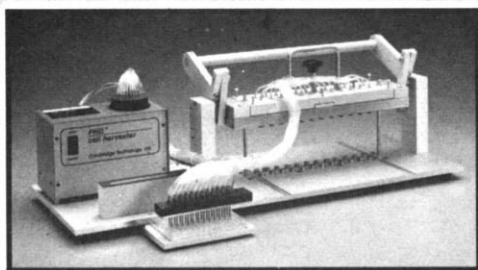
Du Pont has developed an **extraction cartridge for rapid and reliable purification** of nucleic acids. The Nensorb 20 cartridges can purify samples of single- or double-stranded DNA or RNA in a single step taking 15 minutes or less (Reader Service No. 115). Sample size can range from 1 ng to 20 µg with recovery rates of 60 to 90%. Whereas most other solid-phase extraction cartridges are based on ion-exchange resins, the Nensorb 20 contains a proprietary packing material that allows recovery of DNA/RNA in a salt-free water-ethanol solution.

The pick of the literature

Several **new catalogues** have been compiled to tempt the peptide or oligonucleotide researcher. Vega Biotechnologies, Inc. is offering three free chemical catalogues: the *Peptide Synthesis Reagent Catalog*, the *DNA Synthesis Reagent Catalog* and *Bioactive Peptides/Enzymes Substrates/General Biochemicals* (Reader Service No. 116). Sigma Chemical Company offers two new product brochures highlighting their lectins and peptides (Reader Service No. 117). *Lectins from Sigma* describes more than 180 lectins and conjugates with a table on lectin properties and a section on raw material sources. Sigma's new *Bioactive Peptides Catalog* lists over 400 chemicals both by alphabetical order and according to their categories. Amersham International has lumped over 90 of its peptide research products into one listing which groups the products into areas of interest (Reader Service No. 118). A batch-specific analytical data sheet comes free with any order

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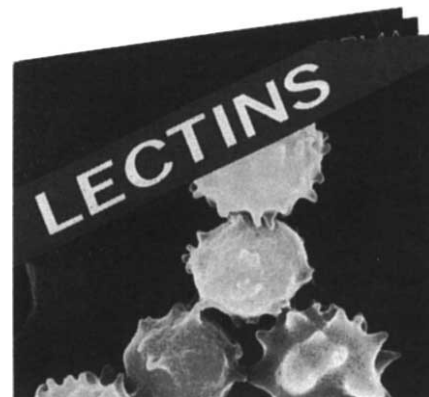
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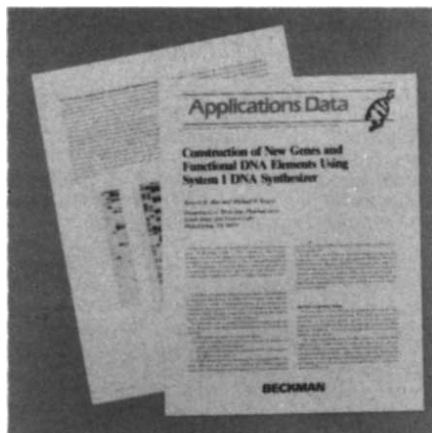


One of the latest mini-catalogues from Sigma

for a Novabiochem catalogue (Reader Service No. 119). This year's updated editions include *Amino Acid Derivatives and Reagents for Peptide Synthesis*, *Biologically Active Peptides*, *¹²⁵I-Labelled Peptides*, *Enzymes and Enzyme Substrates and Enzyme Inhibitors*.

An **applications data sheet** from Beckman Instruments, Inc. describes a gene construction strategy geared to the Beckman System I DNA synthesizer and the

yeast copper-metallothionein (Cu-MT) gene (Reader Service No. 120). Data sheet DS-671, entitled *Construction of New Genes and Functional DNA Elements Using System 1 DNA Synthesizer*, gives

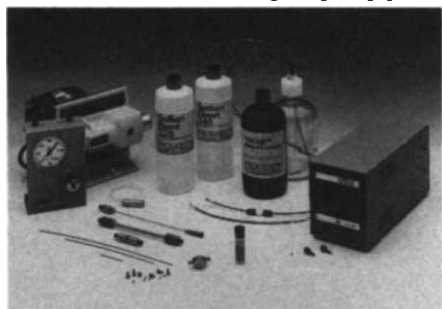


The story of one gene's synthesis

details on selecting sequences for creating sticky ends, unique restriction sites, phosphorylation of the 5' ends and annealing of the DNA fragments to assemble a 105-base-pair portion of the gene.

Analysis...

A new amino acid analysis system for HPLC has arrived from Pickering Laboratories (Reader Service No. 121). The system can be adjusted for either protein/collagen hydrolysates or physiologic fluids and other biological samples. It can be coupled with both integrated liquid chromatographs and component equipment. The product features ion-exchange columns for sodium or lithium chemistries, optimized for regular or high-speed analysis. Two types of guard columns maximize column life. Options available for Pickering Labs' assembly include the CRX390 Post-Column Reactor for high-efficiency ninhydrin development, the FC4870A Flow Conditioner for reagent pump pulse

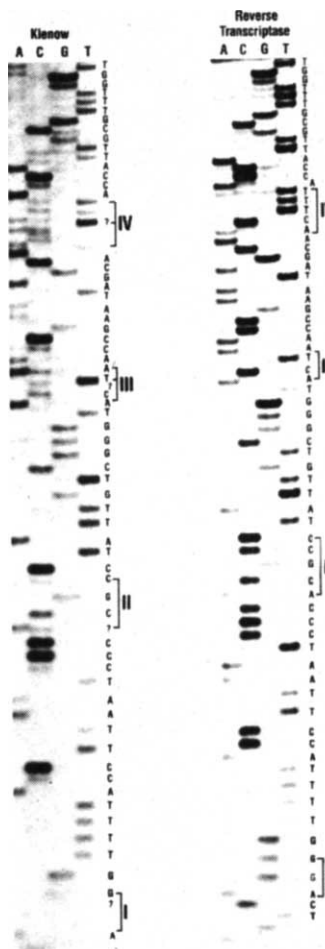


Peptide unpacking gear from Pickering

dampening and the CRX650 Column Temperature Controller. Pickering also supplies OPA and TRIONE reagents and sodium and lithium eluents.

DNA fragments that resist sequencing by DNA polymerase I Klenow fragment may prove more cooperative when subjected to Bio-Rad's M13 Sequencing Kit-

RTase (Reader Service No. 122). The new technique uses **AMV reverse transcriptase for sequencing** by the dideoxy-chain ter-



A Bio-Rad alternative for DNA sequencing

mination method. Bio-Rad's kit also includes deoxy and dideoxy nucleotides, primer No. 1, single-strand sequencing control DNA, reaction buffer, chase solution, stop/load solution, a comprehensive manual and a short protocol. One kit's contents will supply at least 50 sequences.

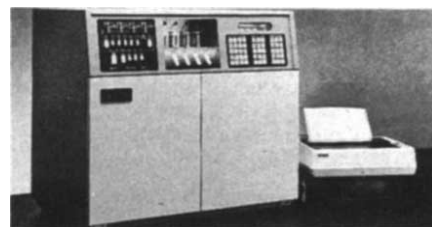
...and synthesis

Vega Biotechnologies, Inc. has devised amino acid-substituted PAM (0CH₂-phenylacetamidomethyl) resins well-suited to automated or manual solid-phase **synthesis of short or long peptide chains** (Reader Service No. 123). Vega determines the substitution of each lot analytically; values usually lie between 0.3 and 0.5 mmole per gram. Vega also confirms particle size, which usually falls between 200 and 400 mesh (polystyrene resin 1% divinylbenzene crosslinked).

Beckman Ltd., Cruachem Inc. and New Brunswick Scientific (U.K.) Ltd. are promoting their **latest in DNA synthesizers**. Beckman's System 1 Plus incorporates the IBM-PC as its interactive graphic controller, using the computer keyboard

or a light pen to enter sequences in the 5' to 3' direction (Reader Service No. 124). Selected bases then appear on the screen, colour-coded and with proper codon breaks. Up to 102 bases can be programmed, and a screen indicates which reagent reservoirs will need refilling before the sequence can be completed. Deviations from the standard program, such as altered pump flow rates or synthesis steps, are effected using a programme adjust.

New Brunswick's model 8600 synthesizer uses both β -cyanoethyl amidite and phosphotriester chemistries for **customizing synthesis cycles** (Reader Service No. 125). With cyanoethyl amidite, fragments in excess of 150 bases with OD 250 or more of product can be produced with cycle time of less than 6 minutes per coupling. Phosphotriester chemistry is appropriate for rapid, economical short-chain synthesis, such as oligonucleotide fragments up to 50 bases long. The phosphotriester cycle time is less than 7

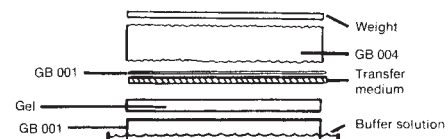


The New Brunswick synthesizer is fully programmable

minutes. With both methods the 8600 offers automated mixed probe-syntheses, automatic de-protection and cleavage and simultaneous four-column synthesis.

The PS 200 from Cruachem Inc. utilizes phosphotriester or phosphoamidite protocols in an **interactive four-column synthesis system** (Reader Service No. 126). Its programmable software issues status reports and directs the operator with screen prompts. A single module houses the valves, controllers and reaction columns. An optional enclosed carousel accommodates up to eight solvent reservoirs.

Schleicher and Schuell have developed four **high-purity grades of blotting paper** as an alternative to the chromatographic grades originally employed in blot transfer techniques (Reader Service No. 127). GB 001, the thinnest blotting paper featured, is intended for protecting gels or cellulose nitrate. GB 004, the thickest paper, has a high absorption capacity to facilitate rapid



A place for blotting paper in transfer assembly and complete transfer as required, for example, with the Southern blot method. □

Astronomy

New Aspects of Galaxy Photometry. J.L. NIETO (ed.). Springer-Verlag:1985. Pp.350. ISBN 0-387-15657-7. Pbk DM 52.

The Sky at Night. By PATRICK MOORE. Patrick Stephens, Wellingborough, UK:1985. Pp.192. Pbk ISBN 0-85059-753-6. £6.99.

Venus, Mars and Satellites of the Outer Planets. R.W. SHORTHILL and A.T. BASILEVSKY (eds). Pergamon:1985. Pp.124. Pbk ISBN 0-08-033197-1. Np.

Psychology

Critical Issues in American Psychiatry and the Law, Vol.2. RICHARD ROSNER (ed.). Plenum: 1985. Pp.307. Hbk ISBN 0-306-41954-8. Np.

The Hands of the Living God: An Account of a Psycho-analytic Treatment. By MARION MILNER. Chatto & Windus: 1985. Pp.444. ISBN 0-7012-0299-8. £17.50.

Psychology. By DIANE E. PAPALIA and SALLY WENDKOS OLDS. McGraw-Hill: 1985. Pp.658. ISBN 0-07-048401-5. £26.25.

When Children Don't Learn; Understanding the Biology and Psychology of Learning Disabilities. By DAVE DIANE MCGUINNESS. Basic Books Inc, New York: 1985. Pp.310 Hbk ISBN 0-465-09178-4. \$19.95.

Sociology

The Custody of Children: A Behavioural Assessment Model. By RICHARD A. MARAFIOTE. Plenum: 1985. Pp.277. Hbk ISBN 0-306-41874-6. Np.

Early Identification of Children at Risk: An International Perspective. WILLIAM K. FRANKENBURG, ROBERT N. EMDE and JOSEPH W. SULLIVAN (eds). Plenum: 1985. Pp.393. Hbk ISBN 0-306-41946-7. Np.

History of Science

From Maxwell to Microphysics: Aspects of Electromagnetic Theory in the Last Quarter of the Nineteenth Century. By JED Z. BUCHWALD. University of Chicago Press: 1985. Pp.339. Hbk ISBN 0-226-07882-5. Np.

The Papers of Joseph Henry, Vol.5, January 1841-December 1843. NATHAN REINGOLD (ed.). Smithsonian Institution Press: 1985. Pp.506. Hbk ISBN 0-87474-793-7. \$45.

The Prehistory of Flight. By CLIVE HART. University of California Press: 1985. Pp.279. ISBN 0-520-05213-7. £29.75, \$40.25.

General

About Science. By BARRY BARNES. Basil Blackwell: 1985. Pp.163. Pbk ISBN 0-631-14158-8. Hbk £16.50; pbk £5.95.

Alternative Therapies. G.T. LEWIS (ed.). Heinemann: 1985. Pp.223. Pbk ISBN 0-433-19270-4. £9.95.

Anticipatory Systems: Philosophical, Mathematical and Methodological Foundations. By ROBERT ROSEN. Pergamon: 1985. Pp.436. ISBN 0-08-031158-X. \$35.

The Art of Planning: Selected Essays of Harvey S. Perloff. LELAND S. BURNS and JOHN FRIEDMANN (eds). Plenum: 1985. Pp.364. Hbk ISBN 0-306-42030-9. Np.

Beningfield's English Landscape. By GORDON BENINGFIELD. Viking: 1985. Pp.140. Hbk ISBN 0-670-80202-6. £14.95.

Benzene: Scientific Update. MYRON A. MEHLMAN (ed.). Alan R. Liss: 1985. Pp.499. ISBN 0-8451-0245-1. £19.

Bergson. By LESZEK KOLAKOWSKI. Oxford University Press: 1985. Pp.115. Hbk ISBN 0-19-287645-7; pbk ISBN 0-19-287644-9. Hbk £8.95; pbk £2.25.

Cambridge and Clare. By HARRY GODWIN. Cambridge University Press: 1985. Pp.230. ISBN 0-521-30765-1. £19.50, \$42.50.

A Century of Controversy: Ethnological Issues from 1860 to 1960. By ELMAN R. SERVICE. Academic: 1985. Pp.351. ISBN 0-12-637380-9. \$48, £42.

Chemie und Gesellschaft: Herausforderung an Eine Welt im Wandel. By G. BOCHE. Pp.215. ISBN 3-921324-01-7. DM 68.

Computational Methods for the Determination of Formation Constants. DAVID J. LEGGETT (ed.). Plenum: 1985. Pp.478. Hbk ISBN 0-306-41957-2. Np.

Creativity in Science — A Symposium Organized by the Fellows of the Los Alamos National Laboratory. MUDUNDI R. RAJU, JAMES A. PHILLIPS and FRANCIS HARLOW (eds). Los Alamos National Laboratory, New Mexico: 1985. Np.

Cultural Imperialism and Exact Sciences. German Expansion Overseas 1900-1930. By LEWIS PYENSON. Peter Lang: 1985. Pp.342. Hbk ISBN 0-8204-0159-5. SWFr 80.

The Cultural-Bound Syndromes: Folk Illnesses of Psychiatric and Anthropological Interest. RONALD C. SIMONS and CHARLES C. HUGHES (eds). D. Reidel: 1985. Pp.516. Hbk ISBN 90-277-1858-X. Dfl.195, £54.25, \$69.00.

Curious Encounters: Phantom Trains, Spooky Spots, and Other Mysterious Wonders. By LOREN COLEMAN. Faber and Faber: 1985. Pp.176. Pbk ISBN 0-571-12542-5. \$11.95.

Current Concepts in Surfactant Research. P. VON WICHERT (ed.). Karger: 1984. Pp.310. ISBN 3-8055-3916-9. DM 269, \$148.

Ethics and Nuclear Arms; European and American Perspectives. RAYMOND ENGLISH (ed.). Ethics and Public Policy Centre, Washington, D.C.: 1985. Pp.144. Pbk ISBN 0-89633-095-8. \$7.

Explosive Remnants of War: Mitigating the Environmental Effects. ARTHUR H. WESTING (ed.). Published for the Stockholm International Peace Research Institute by Taylor & Francis: 1985. Pp.120. Hbk ISBN 0-85066-298-2. £4.95, \$9.

Family and Individual Development. J.A. MEACHAM (ed.). Karger: 1985. Pp.114. Hbk ISBN 3-8055-4037-X. SFr.73, DM 87, \$31.25.

Fluidization, 2nd Edn. J.F. DAVIDSON, R. CLIFT and D. HARRISON (eds). Academic: 1985. Pp.733. ISBN 0-12-205552-7. \$96.50, £75.

Forestry Sciences. Winch and Cable Systems. By IVAR SAMSET. Martinus Nijhoff/Dr W Junk Publishers: 1985. Pp.539. Hbk ISBN 90-247-3205-0. Dfl.175, £60, \$48.50.

Foundations of Business Information Systems. By ANDREW DOSWELL. Plenum: 1985. Pp.221. Pbk ISBN 0-306-41796-0. Np.

A Future for Game? By COLIN MCKELVIE. George Allen & Unwin: 1985. Pp.228. Hbk ISBN 0-04-799030-9. £10.95.

The Future of Piagetian Theory: The Neo-Piagetians. VALERIE L. SHULMAN, LILLIAN C.R. RESTAINO-BAUMANN and LORETTA BUTLER (eds). Plenum: 1985. Pp.222. ISBN 0-306-41940-8. Np.

Gesundheit und Tierschutz. Wissenschaftler melden sich zu Wort. By K.J. ULLRICH and O.D. CREUTZFELDT. ECON Verlag, Dusseldorf, Vienna: 1985. Pp.300. Hbk ISBN 3-430-19229-3. Np.

Glowing Birds: Stories from the Edge of Science. By PATRICK HUYGHE. Faber: 1985. Pp.241. Pbk ISBN 0-571-12533-6. Pbk \$9.95, £5.25.

The Great American Biotic Interchange. FRANCIS G. STEHLI and S. DAVID WEBB (eds). Plenum: 1985. Pp.532. Hbk ISBN 0-306-42021-X. Np.

Handbook of Discourse Analysis. Vol. 3: Discourse and Dialogue. TEUN A. VAN DIJK (ed.). Academic: 1985. Pp.251. ISBN 0-12-712003-3. \$49.50, £40.

Handbook of Discourse Analysis. TUEN A. VAN DIJK (ed.). Academic: 1985. Pp.228. ISBN 0-12-712004-1. \$45.50, £40.

Innovation Policy for Small Farmers in the Tropics: The Economics of Technical Innovations for Agricultural Development. By HANS RUTHERNBERG. Oxford University Press: 1985. Pp.176. ISBN 0-19-859487-9. £17.50.

Localization and Metal-Insulator Transitions. HELLMUT FRITZCHIE and DAVID ADLER (eds). Plenum: 1985. Pp.533. Hbk ISBN 0-306-42077-5. Np.

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Positive Operators. By CHARALAMBOS D. ALIPRANTIS and OWEN BURKINSHAW. Academic: 1985. Pp.367. Hbk ISBN 0-12-050260-7. £51.50, \$59.

Pulse: An Ada-Based Distributed Operating System. By D. KEEFFE, G.M. TOMLINSON, I.C. WAND and A.J. WELLINGS. Academic: 1985. Pp.244. Hbk ISBN 0-12-402970-1. £15.50, \$18.50.

Representation and Responsibility: Exploring Legislative Ethics. BRUCE JENNINGS and DANIEL CALLAHAN (eds). Plenum: 1985. Pp.332. Hbk ISBN 0-306-41994-7. Np.

Return from Death: An Exploration of the Near-Death Experience. By MARGOT GREY. Arkana: 1985. Pp.206. Pbk ISBN 1-85063-019-4. Pbk £4.95.

Reversal Theory: Applications and Developments. M.J. APTER, D. FONTANA and S. MURGATROYD (eds). University College Cardiff Press: 1985. Pp.203. ISBN 0-906449-74-X. £17.50.

Self-Organization: Autowaves and Structures Far from Equilibrium. V.I. KRINSKY (ed.). Springer-Verlag: 1985. Pp.263. ISBN 3-540-15080-3/0-387-15080-3. DM 98.

Shackleton. By RONALD HUNTFORD. Hodder and Stoughton: 1985. Pp.774. Hbk ISBN 0-340-25007-0. £19.95.

The Skywatcher's Handbook: Night and Day. By COLIN A. RONAN. Corgi: 1985. Hbk ISBN 0-552-99196-1; pbk ISBN 0-552-99146-5. Pbk £6.95.

The Structure of Moral Action: A Hermeneutic Study of Moral Conflict. By M.J. PACKER. Karger: 1985. Pp.158. ISBN 3-8055-3999-1. Np.

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Uranium and Nuclear Energy:1984. PROCEEDINGS OF THE NINTH INTERNATIONAL SYMPOSIUM HELD BY THE URANIUM INSTITUTE. The Uranium Institute:1984. Pp.395. ISBN 0-946777-047. £45.

Victimization in Schools. By GARY D. GOTT-FREDSON and DENISE C. GOTTFREDSON. Plenum: 1985. Pp.250. Hbk ISBN 0-306-42023-6. Np.

A Victorian World of Science. By ALAN SUTTON. Adam Hilger, Techno House, Redcliffe Way, Bristol: 1985. Pp.227. Hbk ISBN 0-85274-559-1. £12.50, \$20.

Vietnam Veterans: The Road to Recovery. By JOEL OSLER BRENDE and ERWIN RANDOLPH PARSON. Plenum:1985. Pp.270. ISBN 0-306-41966-1. Np.

Le Vipere d'Italia e d'Europa. By SILVIO BRUNO. Calderini: 1985. Pp.269. Pbk ISBN 88-206-2500-8. Np.

Visions of Apocalypse: End or Rebirth? SAUL FRIEDLANDER, GERALD HOLTON, LEO MARX and EUGENE SKOLNIKOFF (eds). Holmes & Meier, New York: 1985. Pp.268. Hbk ISBN 0-8419-0673-4; pbk ISBN 0-8419-0755-2. Hbk \$28.50; pbk \$15.

Water, Earth, and Fire: Land Use and Environmental Planning in the New Jersey Pine Barrens. By JONATHAN BERGER and JOHN W. SINTON. The Johns Hopkins University Press: 1985. Pp.228. ISBN 0-8018-2398-6. £24.

Women, Drinking and Pregnancy. By MOIRA PLANT. Tavistock: 1985. Pp.168. ISBN 0-422-7810-1. £16.

World Nutritional Determinants. G.H. BOURNE (ed.). Karger: 1985. Pp.225. ISBN 3-8055-3948-7. DM 237, \$118.75.

Archaeology

Ancient Sedimentary Environments (3rd edn.). By R.C. SELLEY. Chapman & Hall: 1985. Pp.317. Pbk ISBN 0-412-25730-0. £10.95.

Beringia in the Cenozoic Era. V.L. KONTRIMACHUS (Editor-in-Chief). Balkema: 1985. Pp.724. ISBN 90-6191-445-0. Dfl.120, \$38.50, £30.

South Atlantic Paleogeography. K.J. HSU and H.S. WEISSERT (eds). Cambridge University Press: 1985. Hbk ISBN 0-521-26609-2. £40, \$69.50.